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Hispanic Foods: Chemistry and Bioactive Compounds

EDITED BY

Michael H. Tunick and Elvira Gonzalez de Mejia

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ACS SYMPOSIUM SERIES **1109**

Hispanic Foods: Chemistry and Bioactive Compounds

Michael H. Tunick, Editor
Dairy and Functional Foods Research Unit
Eastern Regional Research Center
U.S. Department of Agriculture Agricultural Research Service
Wyndmoor, Pennsylvania

Elvira González de Mejía, Editor
Department of Food Science and Human Nutrition
University of Illinois
Urbana, Illinois

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Foreword

The ACS Symposium Series was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of the series is to publish timely, comprehensive books developed from the ACS sponsored symposia based on current scientific research. Occasionally, books are developed from symposia sponsored by other organizations when the topic is of keen interest to the chemistry audience.

Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previous published papers are not accepted.

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Preface

A symposium titled “Chemistry and Flavor of Hispanic Foods” was presented at the Spring National Meeting of the American Chemical Society (ACS) in San Diego in 2005, resulting in an ACS Symposium Series Book, *Hispanic Foods: Chemistry and Flavor*. The success of that symposium and book, along with the continued popularity of and research into Hispanic foods, prompted us to revisit the topic at the ACS meeting in San Diego on March 25-29, 2012, with another “Hispanic Foods” symposium. The presenters, some of whom authored chapters in the first book, kindly contributed to the present volume, *Hispanic Foods: Chemistry and Bioactive Compounds*. As before, the symposium was sponsored by the ACS Division of Agricultural and Food Chemistry, Inc. (AGFD), which is in the forefront in the dissemination of knowledge about food chemistry.

This book includes more on chemistry of Hispanic foods, with an emphasis on compounds that may affect biological processes in humans. Following opening chapters on marketing of these foods in the U.S. and on Queso Fresco, the rest of the chapters discuss the presence and importance of bioactive compounds present in them. The long-term goal is to improve the quality of life through a better nutrition, including in the diet foods, with compounds that will improve human health. The book closes with two chapters on beverages and their importance in the Hispanic diet.

The Hispanic population is increasing in the United States, and the opportunities for producers to provide tasty, nutritious foods to consumers are endless. The presence of compounds with biological properties that go beyond nutrition and reduce the risk of chronic diseases are very attractive for consumers and for the food industry.

As before, we thank our authors for sharing their results with us in the symposium and this book. We also thank AGFD for providing a forum for the symposium and financial support.

Michael H. Tunick
Dairy and Functional Foods Research Unit
Eastern Regional Research Center
U.S. Department of Agriculture, Agricultural Research Service
Wyndmoor, PA 19038
michael.tunick@ars.usda.gov (e-mail)

Elvira González de Mejía, Professor,
Department of Food Science and Human Nutrition
University of Illinois
228 Edward R. Madigan Laboratory
1201 West Gregory Drive
Urbana, IL 61801
edemejia@illinois.edu (e-mail)

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Editors' Biographies

Michael H. Tunick

Michael H. Tunick received a B.S. in Chemistry from Drexel University in 1977. He was a student trainee at the Eastern Regional Research Center of the U.S. Department of Agriculture in Wyndmoor, PA, and was hired as a chemist upon graduation. He performed research on treatment of tannery waste with the Hides and Leather Laboratory until 1983, when he was transferred to what is now the Dairy & Functional Foods Research Unit. He pursued a Ph.D. in Physical-Analytical Chemistry on a part-time basis during this period, receiving the degree from Temple University in 1985. He also became a research chemist in that year and has been involved in a number of projects, including detection of mislabeled cheese, whey protein utilization, investigating characteristics of Hispanic cheese, and development of low-fat Mozzarella for the National School Lunch Program. He currently relates the effects of processing to changes in composition, texture, and microstructure of cheese, and is investigating bioactive compounds in milk from pasture-fed and conventionally-fed cows. He is the Secretary and a Past Chair of the ACS Division of Agricultural and Food Chemistry, has co-edited several ACS Symposium Series books, and was named an ACS Fellow in 2011.

Elvira de Mejia

Elvira de Mejia has a B.S. in Biochemical Engineering, M.S. in Food Science and Technology, and Ph.D. in Plant Biotechnology. She joined the University of Illinois (UI) in 2002 with teaching responsibilities in food chemistry laboratory for undergraduates, and she developed graduate courses in food enzymology and food proteins and enzymes. She has a passion for education and uses research as a teaching tool, focusing on food components with biological benefits to human health and their mechanism of action. She and her team of students have published over 130 peer-reviewed articles and presented over 100 lectures in scientific meetings. She has also served as member of National scientific committees at NSF and USDA. She has mentored students and promoted scientific education internationally. Accomplishments – promoted NSF and USDA funding for underrepresented minorities at UI; fellow of the Mexican Academy of Sciences; received several academic awards for excellence in teaching, research, and international reach.

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Chapter 1

Opportunities and Challenges for the Marketing of Hispanic Foods in the United States

Luis Antonio Mejia, Ph.D.[*]

Adjunct Associate Professor, Department of Food Science and Human Nutrition, University of Illinois, Urbana-Champaign, Bevier Hall, 905 S. Goodwin Ave., Urbana, IL 61801
[*]E-mail: lamejia@illinois.edu

The Hispanic food and beverage market is growing significantly and represents an economically important food trend in the United States. This market is primarily driven by taste but nutritional and health benefits may play an important marketing role in the future. Contributing factors for the growth of the Hispanic food and beverage market include the growing Hispanic population, the increasing purchasing power of the Hispanics and their emotional desire for the taste of their motherlands. Availability of Hispanic foods and beverages in the United States comes from: a) food distributors of foods and food ingredients imported from Latin American countries and, b) U.S. food manufacturers using mostly imported Latin American raw materials. There are opportunities to grow this market further by expanding the commercialization of existing products and the introduction of novel ethnic foods. Challenges that need to be addressed include the marketing to a sub-segmented Hispanic market distributed in the country regionally different, regulatory compliance for new product introductions and the development of the appropriate technology to industrialize novel foods and ingredients.

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Introduction

The size of the Hispanic food and beverage market in the United States has become significantly important and continues to grow rapidly. Based on market research data from 2010, the estimated size of this market segment is about $7 billion, up 29 % from $5.4 billion in 2005 (*1*). Furthermore, it has been predicted that the Hispanic food and beverage market will reach approximately $10 billion in 2014 (*1*). With all the varieties of foods and flavors of Latin American origin and their increasing acceptance by the U.S. population, there is no doubt that Hispanic foods and beverages represent an important ethnic food trend in the U.S. (*2*).

In the general population, including both Hispanics and American consumers, the Hispanic food and beverage market is driven primarily by taste. However, there are other important contributing factors that need to be considered and which represent attractive opportunities for expanding the Hispanic food and beverage business.

Main Contributing Factors for the Growth of the Market Population Dynamics

The rapidly growing Hispanic population parallels the growth of the market. This is an important opportunity for expansion. According to the latest 2010 U.S. Census (*3*), the current total population of the United States is 308, 745, 538 inhabitants (Table I). Out of this total, 50,447,594 are individuals of Hispanic origin, representing 16.3% of the population. In comparison with a similar census of 2000, the Hispanic population grew on average 43% while the total U.S. population grew only 10%. Based on this new census data, the Hispanic population now ranks as the largest minority residing in the U.S. Individuals of Mexican origin comprise by far the largest segment (63%), followed by Puerto Ricans (9.2%), Central Americans (7.9%), South Americans (5.5%), Cubans (3.5%) and Dominicans (2.8%). Although still relatively small markets in comparison with the Mexican segment, the most rapidly growing Hispanic groups are the Central Americans (growth rate 137%, primarily Salvadorians followed by Guatemalans and Hondurans) and the South Americans (growth rate 104%, primarily Colombians, Ecuadorian and Peruvians). In absolute numbers, however, the Mexican segment with a growth rate of 54% is the largest growing Hispanic group. As shown in Table I, the Hispanic group of Mexican origin gained from 2000 to 2010 about 11.2 million new members, while other groups, like the fastest growing Central American group, grew only 2.3 million people during the same time period. This demographic distribution is important because individuals from different origins have different food and culinary cultures. While Mexicans may love to eat nopalitos (young cladodes of Nopal, *Opuntia sp.*), Puerto Ricans will prefer mofongo (mashed plantains and pork cracklings), Salvadorians pupusas (thick handmade corn tortilla filled with cheese, beans, pork meat or a combination thereof), and Colombians arepas (corn cakes made from pre-cooked corn flour). This cultural segmentation may represent a challenge for the marketing of Hispanic foods and beverages because people from different Hispanic or Latino origin do not reside homogeneously in the territorial U. S., but is concentrated in different regions or states. As illustrated in Table II, the state distribution of different Hispanic groups, of one million or more, varies depending on their origin. As indicated by their respective rankings in the table, Mexicans reside mainly in California, Texas and Arizona; Puerto Ricans in New York, Florida and New Jersey; Cubans in Florida, California and New Jersey; Salvadorians in California, Texas and New York; Dominicans in New York, New Jersey and Florida and Guatemalans in California, Florida and New York (*3*). Understanding this distribution may optimize the food distribution and marketing efforts.

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Table I. Hispanic Population and its Growth between 2000 and 2010. Data are from Reference (*3*)

	2000		***2010***		***Growth Rate***
U.S. Population	**281,421,906**	**100%**	**308,745,538**	**100%**	**10%**
Hispanics	**35,305,818**	**12.5%**	**50,447,594**	**16.3%**	**43%**
Mexican	20,640,771	58.5%	31,798,258	63%	54.1%
Puerto Rican	3,406,178	9.6%	4,623,716	9.2%	35.7%
Central American	1,686,937	4.8%	3,998,280	7.9%	137%
South American	1,353,562	3.8%	2,769,434	5.5%	104.6%
Cuban	1,241,685	3.5%	1,785,547	3.5%	43.8%
Dominican	764,945	2.2%	1,414,703	2.8%	84.9%

Purchasing Power of Hispanics

Another important opportunity, despite the deceleration of the U.S. economy in the last few years, is the increasing purchasing power of Hispanics. As indicated in Table III, the buying power of Hispanics in the U.S. increased from $210 billion in 1990 to $1 trillion in 2010 and has been estimated to reach $1.5 trillion in 2015 (*4*). Between 2000 and 2010 the data represent a growth rate of 108%; a significantly higher rate than the 48% for the non-Hispanic population. The share of buying power, although still relatively low, also increased for the Hispanic population from 5% in 1990 to 9.3% in 2010 (*4*). This increase in buying power of Hispanics can be related in part to a higher educational level achieved by young U.S. born Hispanics entering the work force in the last few years (*3*, *4*). It is also important to note, when considering this higher buying power, that Hispanics households spent relatively more on foods (restaurants and groceries) than no-Hispanics (*4*, *5*).

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Table II. State Distribution of Hispanic Groups with Population sizes of more than One Million. Data are from Reference (*3*)

Origin	***Total U.S.***	***Rank 1st***	***Rank 2nd***	***Rank 3d***	***Rank 4th***	***Rank 5th***
Mexican	31,798,258	CA; 11,423,146	TX; 7,951,193	AZ; 1,657,668	IL; 1,602,403	CO; 759,181
Puerto Rican	4,623,716	NY; 1,070,558	FL; 847,550	NJ; 434,092	PA; 366,082	MA; 266,125
Cuban	1,785,547	FL; 1,231,438	CA; 88,607	NJ; 83,362	NY; 70,803	TX; 46,541
Salvadorian	1,648,968	CA; 573,956	TX; 222,599	NY; 152,130	VA; 123,800	MD; 123,789
Dominican	1,414,703	NY; 674,787	NJ; 197,922	FL; 172,451	MA; 103,292	PA; 62,348
Guatemalan	1,044,209	CA; 332,737	FL; 83,882	NY; 73,806	TX; 66,244	NJ; 48,869

Table III. Buying Power of Hispanics. Data are from Reference (*4*)

	1990	*2000*	*2010*	*2015*	*% Diff. 2000-2010*
Buying Power	$ 210 billion	$ 499 billion	$ 1 trillion	1.5 trillion	108 % *
Market Share	5%	6.8 %	9.3%	----------	

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The Cultural Heritage

An additional consideration to further grow the food and beverage Hispanic market is to leverage the cultural and emotional traditions that are brought to the U.S. by Hispanics from their native countries. Hispanics residing in the U.S. are more inclined to conserving their traditions of having more home family meals using ingredients from their countries of origin. In addition, they are seduced by the flavors of the foods they enjoyed as youths and the “nostalgia” of their own land and family traditions. Another factor that has contributed with the growth of Hispanic foods in the U.S. is the acceptance and adoption of Latin foods by Americans, mainly driven by taste, fun and enjoyment. Now main stream processed foods in the market like salsas, tortilla chips, nachos, quesadillas, cooked beans and taco shells can be found in practically any grocery store throughout the country and are often part of the U.S. diet. In addition, fruit and vegetables from Latin America such as mangos, pineapples, bananas, avocados, and chili peppers are commonly present on the U.S. table. Furthermore, Latin spices such as oregano and herbs such as cilantro are also common ingredients of U.S. recipes.

Availability of Hispanic Foods in the U.S.

Beyond main stream Hispanic products like tortillas, nachos, salsas, guacamole and other Mexican type products offered by fast food restaurants, the more authentic Hispanic foods are either imported from Latin American countries, including the Caribbean or manufactured in the U.S. by American companies using Latin American raw materials and traditional recipes. Imported processed foods in the U.S. market include tamales, refried beans, cooked plantains, nopalitos (*Opuntia, sp*), fried cassava, panela, arepas, dulce de leche, coconut milk, mango pulp, etc. Even Coca Cola is imported into the U.S. from Mexico because it is believed to taste differently from the U.S. version. The availability of Hispanic foods in the U.S. comes from two main sources: a) food distributors located in different strategic U.S. regions which import Latin Foods from different Latin American countries and b) food processing or packing U.S. companies which manufacture Hispanic foods in the U.S. using mainly imported Latin American ingredients. Table IV shows examples of U.S. distributors of

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Hispanic foods such as Dismex (Miami, FL), Marcas Foods (Franklin Park, IL), Diaz Foods (Atlanta GA), Intermark Foods (Doral, FL), Novamex (El Paso, TX) and there are even internet distributors like Amigo Foods (Amigosfoods.com, Miami, FL) that sell on-line traditional Latin foods from the Latin American country of your choice. On the other hand, Mexican food manufacturers in the U.S. have been in existence for many years and represent the cornerstone of Hispanic foods produced or packed in the U.S. These manufacturing companies are located primarily in California and Texas where the majority of Hispanics of Mexican origin reside. Examples are Juanitas Foods (Wilmington, CA) which manufactures and commercializes traditional ethnic Mexican foods like menudo, pozole, fajitas, carnitas and mole; Ruiz Foods (Dinuba, CA) which produces frozen Mexican foods including frozen burritos, chimichangas, quezadillas and tamales; Mission Foods (Irving, TX) , which is one of the major manufacturers of corn, wheat and multigrain tortillas using even ancient grains such as amaranth (*Amaranthus, spp*), quinoa (*Chenopodium, spp*) and sorghum (*Sorghum, spp*) and Old El Paso (Owned by General Mills, Minneapolis, MN) which produces Mexican dinner kits, “Heat and Serve” Mexican meals, canned jalapeno peppers, refried beans, enchilada sauces, taco shells and dips. More recently the Mexican company Bimbo entered the U.S. market under the name of Bimbo Bakeries (Horsham, PA) producing bread, pastries and tortillas. Most innovative, in terms of new product introductions using a wider Latin American scope is Goya Foods (Secaucus, NJ). This company packs and commercializes throughout the U. S., beans, rice, condiments, beverages, frozen foods and regional specialties of Caribbean, Mexican and Central American origin. Besides the more traditional ethnic Latin Foods, Goya Foods packs and commercializes more exotic ethnic food items such as pacaya (*Chamaedorea tepejilote*), nance (*Byrsonima crassifolia*), loroco (*Fernaldia pandurata*), chiltepe (*Capsicum annum var. glabriusculum*), tecojotes (*Crataegus Mexicana*) and the corn-fungus huitlacoche (*Ustilago maydis*).

Table IV. Examples of Companies which Commercialize Hispanic food/food ingredients in the U.S.

Distributors/Importers	***Manufacture/Packagers***
Dismex, Miami, FL. Distributes Mexican and Central American specialty products. Include brands such as Boing, Del Frutal, Diana, Dona Maria, San Marcos, Herdez, Jumex, La Costena, Maseca, INA, Del valle, Gamesa, El Paraiso. www.dismexfood.com	- **Juanitas Foods, Wilmington, CA**. Ethnic Mexican foods like menudo, pozole, fajitas, carnitas, mole. www.juanitasfoods.com
- **Marcas Food Distributor, Franklin Park, IL**. Commercializes Latin Foods under the private label of “Mareli Brand” (e.g. plantain chips, yuca chips,	- **Ruiz Foods, Dinuba, CA**. Produces a line of frozen Mexican Foods under the “El Monterey” brand; includes products

Continued on next page.

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Table IV. (Continued). Examples of Companies which Commercialize Hispanic food/food ingredients in the U.S.

Distributors/Importers	*Manufacture/Packagers*
piloncillo, dulce de leche, guayaba, nance, chicharon, pacaya, sugar cane, chiltepe). www.marelifoods.webs.com	as burritos, quezadillas and tamales. www.elmonterey.com
- **Diaz Foods, Atlanta, GA**. Stocks an extensive selection of food products from Mexico, Central and South America and the Caribbean. www.diazfoods.com	- **Mission Foods, Irving, TX**. Major manufacturer of tortillas (corn, wheat and other), taco shells, salsas, guacamole dip. www.missionfoods.com
- **Intermark Foods, Doral, FL**. Distributes regional Latin American specialties, including cheeses, cheese breads (pan de bono), corn breads (arepas), tropical fruit smoothies, frozen fruits and vegetables and processed meats. www.intermarkfoods.com	- **Old El Paso (Owned by General Mills, Minneapolis MN).** Mexican dinner kits, ready to heat and serve Mexican meals, refried beans, enchilada sauces, taco shells, salsas, dips. www.oldelpaso.com
- **Novamex, El Paso, TX.** Distributes Mexican beverages such as Sangria Senorial, Jarritos, etc. www.novamex.com	- **Bimbo Bakeries, Horsham, PA**. Bread, pastries, tortillas. www.bimbobakeries.com
- **Amigo Foods.** Internet distributor of a variety of local foods from Latin American countries, including products as pupusas, tamales, etc... www.amigofoods.com	- **Goya Foods, Secaucus, NJ.** Rice, beans, condiments, beverages, frozen foods and a large variety of regional specialties from the Caribbean, Mexico and Central American, including quinoa, chiltepe, pupusas, loroco, nance, yuca, pacaya and coconut water. www.goya.com

Potential Nutritional and Health Benefits of Hispanic Foods

Less commercially exploited has been the nutritional contribution and potential health benefits of Hispanic foods. These foods can be important sources of micronutrients and bioactive compounds that can be linked to prevention of certain disease conditions. However, using this marketing approach could be a real challenge because this is a segment strongly driven by taste. However, with time, consumers can be educated on scientifically well-supported health claims. Thus, it is necessary to explore further the relationship between Hispanic foods and the epidemiological profile of the Hispanic population. For example, the prevalence of diabetes is particularly high among Hispanics in the U.S. and there may be bioactive compounds in Latin-origin foods which may contribute with its prevention and/or treatment. According to the National Institute of Diabetes and Digestive and Kidney Diseases, based on 2007-2009 data, the prevalence of diabetes among Hispanics 20 years of age or older is 11.8%, while the prevalence in non-Hispanics is only 7.1% (*6*). Foods of Latin origin which have been linked to the management of diabetes include, for example, nopal (young cladodes

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of *Opuntia spp* known as nopalitos), a traditional food widely consumed in Mexico. A recent study conducted by the National Autonomous University of Mexico has demonstrated that consumption for a period of two months of a diet that includes nopal (*Opuntia sp*) combined with other local Mexican foods can reduce the levels of serum triglycerides and glucose intolerance in patients with metabolic syndrome (*7*). A broader discussion on the potential effects of *Opuntia, spp* on diabetes and other disease conditions is presented in this book. In addition, “flor de Jamaica” (*Hibiscus sabdariffa*), used to prepare a popular infusion widely consumed in Mexico and other Central American and Caribbean countries, either as hot tea or as a cold beverage known as “agua de Jamaica”, has been related to the control of hypertension (*8*). A more extensive discussion on the nutraceutical effects of *Hibiscus sabdariffa* is presented in Chapter X. Other potential benefits of several Hispanic foods and beverages are discussed in the various chapters of this publication. Research is needed to explore and develop this health/nutrition platform of Hispanics foods so that it can contribute to both, expanding the Hispanic food and beverage market and, at the same time benefit not only Hispanics but also the U.S. population at large.

Conclusion

In perspective, the growing Hispanic population and the adoption of Hispanics foods by the U.S. population as a whole represent attractive opportunities for the food industry to continue expanding the growth of the Hispanic food and beverage market. Besides flavor, the nutritional and potential health benefits of foods of Latin origin should be considered. In doing so, there will be challenges that need to be addressed and resolved to optimize marketing success. These challenges will include the identification of novel bioactive compounds and for each such compound, determine its potential health benefits, obtain its regulatory clearance and to develop appropriate technologies for their industrialization.

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Chapter 2

Chemistry of Queso Fresco

Michael H. Tunick,*,[1] Diane L. Van Hekken,[1] Ling Guo,[2] and Peggy M. Tomasula[1]

**[1]U.S. Department of Agriculture, Agricultural Research Service,
Eastern Regional Research Center, Dairy & Functional Foods Research Unit,
600 E. Mermaid Lane, Wyndmoor, PA 19038
[2]Present address: Key Laboratory of Dairy Science, Northeast Agricultural University, No. 59 Mucai St., Xiangfang District, Harbin, P.R. China 150030
*E-mail: Michael.Tunick@ars.usda.gov**

Queso Fresco (QF), the most popular Hispanic cheese in the U.S. and Mexico, was fully characterized in a series of studies intended to improve quality traits. QF is a non-melting variety made with rennet and without starter culture, and the curd is commonly finely milled to ensure a crumbly texture. In northern Mexico, QF is traditionally made from raw milk and has more moisture and less NaCl than QF made in the U.S. from pasteurized milk. Although QF normally contains up to 3% NaCl, lowering the NaCl content to 1.5% does not substantially alter its characteristics. During storage, activity of spoilage bacteria contributes to proteolysis, appearance of volatile compounds presumably generated from lipolysis, and decreases in lactose and pH levels, though the pH stays over 6.0. Texture, melt, and microstructure do not change appreciably over 8 wk of storage, the typical shelf life for QF in the U.S., though shear strain and color intensity do change under certain conditions. A full knowledge of the qualities of QF will enable cheesemakers to create a better product.

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Introduction

A number of Hispanic dairy products were described in the third chapter of the 2007 ACS Symposium Series Book *Hispanic Foods: Chemistry and Flavor* (*1*). In the present chapter, we focus on one of these products, Queso Fresco (QF). This popular cheese variety is coagulated with rennet, cooked at 30-32°C, drained of whey, salted directly, and finely milled to obtain a crumbly, non-melting product. It differs from the acid-set Queso Blanco, which is traditionally set with lemon juice or vinegar and is not milled (*2*).

The characteristics of QF have been investigated by the Dairy & Functional Foods Research Unit (DFFRU) over the past few years to develop guidelines for the industry and the National School Lunch Program (*3*–*7*). The research began with a survey of commercial cheeses made in Mexico from raw milk (RM) and in Mexico and the U.S. from pasteurized milk (PM) to identify desirable traits (*3*, *4*). The effects of storage temperature (4 or 10°C) on the cheese were investigated since many home refrigerators, warehouses, and delicatessen dairy cases may be above 4°C (*6*). A study on the impact of reducing NaCl content in QF was also conducted since health organizations recommend that Americans significantly decrease sodium and salt intake (*5*, *7*). This chapter details DFFRU research on composition, volatile compounds, microbiology, protein profiles, texture, rheology, color, melting, microstructure, and sensory attributes of QF made and stored under various conditions.

Materials and Methods

Commercial Cheeses

In the commercial cheese survey, QF samples were obtained from large-scale and artisanal Mexican manufacturers in the Hermosillo, Sonora area. Six cheesemakers each supplied three 2-kg blocks from three different batches of cheese within a day of manufacture. These included four RM and two PM brands, and were analyzed within 10 d of manufacture. In addition, PM cheeses from U.S. plants in California, Illinois, North Carolina, and Wisconsin were obtained at grocery stores. Two packages of each, weighing 0.35-0.45 kg, were analyzed approximately 30 d before their sell-by dates.

Cheese Preparation

The remainder of the cheeses in these studies was manufactured in the DFFRU laboratory using the following procedure (*5*–*7*). Milk obtained from a local dairy was standardized at 3.5% fat and pasteurized at 72°C for 15 s. The milk was homogenized in two stages (6.9 and 3.4 MPa) and 180 kg containing 180 g of added $CaCl_2$ was adjusted to 32°C in a stainless steel vat. No starter culture was added. The milk was coagulated with chymosin (14 mL Chy-Max, Chr.

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Hansen, Milwaukee, WI; diluted in 200 mL water) and cooked at 39°C for 30 min. Approximately 90 kg whey was drained and the curd was wet salted in three applications. The rest of the whey was drained and the curds were chilled to 21°C. After finely milling into small pieces with a grinder (Bosch universal kitchen machine, Robert Bosch Hausgeräte, Dillingen, Germany), the cheese was hand-packed into molds for storage overnight at 4°C. The next day, each cheese was removed from its mold, sliced into three blocks, and vacuum packaged. Samples were stored at 4 or 10°C for up to 8 wk. The NaCl level of most of the cheeses was targeted at 2.5% w/w, but percentages of 1.0, 1.5, and 2.0 were used in the NaCl content study. Consumer sensory studies of 2.5% NaCl QF showed that the DFFRU cheese was liked and often could not be distinguished from commercial QF by untrained panelists (*6*).

Composition

Moisture was determined by the forced-draft oven method using AOAC Official Method 948.12 (*8*). Protein was calculated by EA1112 nitrogen analyzer (CE Elantech, Lakewood, NJ) with the N_2 result multiplied by 6.38. Lactose was determined by YSI 2700 biochemistry analyzer (YSI Inc., Yellow Springs, OH) and pH by Orion model 611 pH meter (Orion Research Corp., Cambridge, MA). These analyses were performed in triplicate. Fat was analyzed in duplicate by the Babcock method (*9*) and NaCl was determined in duplicate by chloride titrator strip (Hach Co., Loveland, CO) using AOAC Official Method 971.19 (*8*).

Other Analyses

Volatile compounds were identified by GC-MS using the procedure of Tunick et al. (*6*). Aerobic bacteria plate counts were determined following standard microbiological procedures for dairy products (*10*) using the procedure by Renye et al. (*3*). Protein profiles were obtained by SDS-PAGE using the procedures of Van Hekken et al. (*11*) and Tunick et al. (*12*). Texture profile analysis (TPA), small amplitude oscillatory shear analyses (SAOSA), and torsion gelometry were conducted as previously described (*13*). Melt and color properties were investigated based on the procedures described by Olson et al. (*14*) as modified by Guo et al. (*5*). Meltability was determined in triplicate using the Schreiber Melt Test as described by Kosikowski and Mistry (*9*). Scanning electron microscopy (SEM) was conducted as previously described (*15*).

Statistics

Analysis of variance with mean separation using the LSD technique (*16*) was used for statistical analyses. Differences are described as significant only when $P < 0.05$.

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Table I. Composition of Queso Fresco in study of cheeses made from raw milk (RM) and pasteurized milk (PM), and in studies of storage temperature and NaCl content

	Moisture	*Fat*	*Protein*	*NaCl*	*Lactose*	*pH*
			(%)			
			Commercial sample study			
Mexican RM	58.9[a]	21.4[b]	16.8[a]	1.02[b]	nd[1]	5.43[ab]
Mexican PM	49.4[b]	31.0[a]	18.4[a]	1.13[b]	nd	5.26[b]
US PM	50.6[b]	25.0[b]	19.7[a]	1.63[a]	nd	6.04[a]
			Storage temperature study			
1 wk at 4°C	56.2[ab]	21.8[a]	15.2[a]	2.48[a]	2.71[a]	6.34[a]
8 wk at 4°C	56.7[ab]	21.5[a]	15.1[a]	2.13[b]	2.68[a]	6.32[a]
1 wk at 10°C	56.9[a]	22.1[a]	14.7[b]	2.48[a]	2.73[a]	6.32[a]
8 wk at 10°C	54.9[b]	22.1[a]	15.0[a]	2.12[b]	2.45[b]	6.10[b]
			NaCl content study			
1.0%[2] , 1 d	56.6[a]	22.6[a]	17.9[a]	1.28[b]	2.97[a]	6.32[a]
1.0%, 8 wk	55.2[a]	23.2[a]	16.9[b]	1.16[b]	2.47[c]	6.11[b]
1.5%, 1 d	55.9[a]	23.3[a]	17.4[b]	1.85[ab]	2.81[b]	6.30[a]
1.5%, 8 wk	55.0[a]	23.1[a]	17.3[b]	1.78[ab]	2.47[c]	6.10[b]
2.0%, 1 d	56.4[a]	22.2[a]	17.1[b]	2.15[a]	2.86[b]	6.28[a]
2.0%, 8 wk	55.0[a]	22.3[a]	17.2[b]	2.09[a]	2.51[c]	6.09[b]

[abc]In each study, means within the same column with different subscripts are different ($P < 0.05$). [1] nd = not determined. [2] Percent NaCl.

Results and Discussion

Composition

The average composition of commercial QF samples and cheeses prepared by DFFRU is shown in Table I. The RM cheeses contained significantly more moisture and less fat than the Mexican PM cheeses; protein, NaCl, and pH were not different. The higher moisture in the RM cheeses was due to preferences by the cheesemakers, who adjust moisture levels by cutting the curd into larger pieces, using lower cooking temperatures, or use shorter cooking times. The PM cheeses from the US had more NaCl than the Mexican cheeses and were similar to Mexican RM samples in fat, protein, and pH. The differences in NaCl content were also a choice by the manufacturers.

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In the storage temperature and NaCl content studies, the goal was to produce QF with around 56% moisture and 22% fat. The initial NaCl content in the temperature study was 2.5%. The values for moisture, fat, and protein remained the same during storage in almost all cases; some NaCl was lost to the whey in the storage temperature study but not in the NaCl content study. The values for lactose and pH significantly decreased with aging in most cases, indicating that microorganisms were metabolizing lactose into lactic acid. The pH remained above 6.0 in all of the cheeses.

Volatile Compounds

GC-MS data showed that volatile lactones, ketones, alcohols, and aldehydes were generated during the storage temperature study. The methyl ketones 2-heptanone and 2-nonanone and nonalactone were detected at each storage time, and δ-dodecalactone was observed at 4 and 8 wk. Samples stored at 10°C tended to contain more of these. 3-Methyl-1-butanol, phenyl ethyl alcohol, and 2,3-butanediol were detected in the 10°C samples after 4 wk and were present in all samples at 8 wk. Nonanal and decanal appeared in every sample at every storage time. Pentanal, hexanal, and heptanal were observed in the cheeses at 1 and 4 wk only, presumably degrading into simpler compounds after that. Phenylacetaldehyde and 3-methyl butanal appeared after 4 and 8 wk in the 10°C samples, but not until 8 wk in the 4°C cheeses. The higher storage temperature appeared to enhance aldehyde formation and degradation.

Acetic acid and a number of the volatile fatty acids often observed found in cheese were also found. Acetic acid was observed at 4 and 8 wk, and octanoic, decanoic, and dodecanoic acids were found throughout. Though butanoic and hexanoic acids were not observed, an ester of propionic acid was detected throughout, and nonanoic acid appeared at 8 wk. The presence of volatile fatty acids indicated that lipolysis was taking place in the cheeses. Aside from free fatty acids, the major products of lipolysis include aldehydes from cleavage of unsaturated fatty acids, lactones from breakdown of hydroxyacids, and methyl ketones and secondary alcohols from degradation of β-ketoacids (*17*). All of the cheeses contained representatives from these classes of compounds. The pasteurization of the cheesemilk should have inactivated any indigenous lipolytic enzymes, and starter culture and lipase were not added, so the lipolysis presumably arose from either the chymosin or from spoilage microorganisms. Species of *Pseudomonas* have lipolytic activity in dairy products (*18*) and the chymosin used in this study does not, indicating that spoilage microbes were responsible for lipolysis in the cheese.

Microbiology

Pasteurized milk was used to make the DFFRU cheese, so the total aerobic population growth in the fresh QF was low, ranging from 1.63 $\log_{10}$ to 2.67 $\log_{10}$ cfu g^{-1}. Spoilage bacteria are ubiquitous in the environment, and after 8 wk of storage the total aerobic population increased significantly in the QF containing 1.0% NaCl (from 2.25 $\log_{10}$ to 5.45 $\log_{10}$ cfu g^{-1}), 1.5% NaCl (from 2.67 $\log_{10}$ to

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5.46 $\log_{10}$ cfu g^{-1}), and 2.0% NaCl (from 2.03 $\log_{10}$ to 5.47 $\log_{10}$ cfu g^{-1}). Artisanal RM QF contains high number of mesophilic bacteria (7.31 $\log_{10}$ to 8.98 $\log_{10}$ cfu g^{-1}) within 1 wk of manufacture (*3*). The Mexican government recommends a maximum of 5.70 $\log_{10}$ cfu g^{-1} for cheese (*19*).

By 8 wk, there were a number of small and yellow colonies (identified as *Microbacterium oxydans*), medium and white colonies (*Paenibacillus polymyxa* and *Pseudomonas mandelii*), and large and white colonies (*Pseudomonas putida*). These bacteria are spoilage organisms commonly found in dairy products: strains of *Microbacterium* and *Paenibacillus* are thermoduric bacteria often isolated from processed milk products (*20*), and *Pseudomonas* species tend to be isolated most often. *Pseudomonas* are predominantly responsible for limiting the shelf life of processed refrigerated fluid milk, and may present quality concerns (*21*).

Table II. Concentration (% wt/wt) of caseins (CN) and protein fragments in Queso Fresco

	Caseins				*Protein fragments (kDa)*			
	α_{s2}-CN	*α_{s1}-CN*	*β-CN*	*para-κ-CN*	*22*	*22-18.5*	*18-15*	*<14*
	Storage temperature study							
1 wk, 4°C	7.5^{a}	38.0^{a}	32.4^{a}	10.4^{a}	0.0^{c}	4.1^{b}	3.0^{a}	2.6^{c}
8 wk, 4°C	6.2^{a}	29.4^{ab}	19.4^{b}	11.4^{a}	8.3^{b}	11.6^{a}	4.9^{a}	7.6^{b}
1 wk, 10°C	7.0^{a}	36.1^{a}	31.0^{a}	11.2^{a}	0.0^{c}	5.5^{b}	3.5^{a}	3.8^{c}
8 wk, 10°C	6.1^{a}	23.1^{b}	15.8^{b}	12.6^{a}	12.0^{a}	12.8^{a}	4.7^{a}	11.6^{a}
	NaCl content study							
1.0%[1], 1 d	10.0^{a}	33.9^{a}	31.6^{a}	9.8^{a}	0.0^{b}	4.2^{b}	1.5^{b}	3.1^{b}
1.0%, 8 wk	8.3^{ab}	24.4^{b}	14.4^{b}	11.2^{a}	7.1^{a}	13.7^{a}	4.6^{a}	9.4^{a}
1.5%, 1 d	10.0^{a}	33.4^{a}	31.3^{a}	9.9^{a}	0.0^{b}	4.4^{b}	1.5^{b}	3.3^{b}
1.5%, 8 wk	8.2^{ab}	25.7^{b}	19.1^{b}	10.7^{a}	5.3^{a}	10.9^{a}	3.7^{ab}	10.1^{a}
2.0%, 1 d	9.8^{ab}	34.1^{a}	33.0^{a}	9.8^{a}	0.0^{b}	3.3^{b}	1.3^{b}	3.0^{b}
2.0%, 8 wk	7.9^{b}	25.3^{b}	19.0^{b}	11.0^{a}	6.4^{a}	10.5^{a}	3.6^{ab}	9.7^{a}

^{abc}In each study, means within the same column with different subscripts are different ($P < 0.05$). [1] Percent NaCl.

Protein Profiles

During storage, the levels of α_{s1}- and β-caseins decreased while the levels of casein fragments increased (Table II). α_{s1}-, β-, and κ-caseins are degraded by chymosin in rennet, and plasmin, an indigenous milk proteinase, breaks down α_{s1}-, α_{s2}-, and β-caseins (*22*). The α_{s1}-casein decreased by one-fourth in the 4°C cheeses and by one-third in the 10°C cheeses during 8 wk of storage. The levels of β-casein during storage went down by two-fifths at 4°C and by a half at 10°C. Similar decreases were also seen in the NaCl study, where the NaCl content did not affect the extent of proteolysis. The para-κ-casein level held steady, giving the appearance of an increase in relation to the other intact caseins. Since no starter culture was used, the results indicate that the increased proteolysis of casein at 10°C was due to spoilage bacteria.

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Texture

TPA showed that the U.S. PM cheese was the hardest and least cohesive of the commercial samples (Table III). Cheese manufacture is tailored toward consumer preference, and cheesemakers in the U.S. evidently feel that their QF should be harder and more crumbly than the Mexican versions. QF is milled to break up the protein matrix, resulting in a crumbly texture. The hardness values were much lower than those of previously tested cheeses such as Brick, Cheddar, Colby, Gouda, and Mozzarella, whose values ranged from 35 to 89 N (*13*). The range of cohesiveness values was not far from those of Cheddar and Colby (0.13-0.28), but was lower than those of the more resilient Gouda and Mozzarella (0.34-0.62). Hardness and cohesiveness were unaffected by NaCl content, storage temperature, or storage time.

Rheology

The SAOSA results are shown in Table III. G′ is a measure of the energy stored during an oscillation and G″ is a measure of the energy lost. Lower values are indicative of a weak casein matrix, and except for the U.S. PM cheese the G′ and G″ values for QF are less than those for other varieties previously tested (*13*). The values did not vary with NaCl or storage.

Table IV shows the torsion analysis results. The shear stress and shear strain values were smaller than those of less crumbly and more flexible cheeses such as Brick, Gouda, and Mozzarella, whose shear stress values are 22-56 kPa and shear strain values are 1.3-1.9 (*13*). The force and deformation required to fracture QF are low because of the discontinuous nature of its protein matrix. Moisture content also plays a role: the Mexican RM cheese had a high moisture level and low shear stress, and the Mexican PM cheese had low moisture and high shear stress. The shear strain increased with time in the storage temperature study. These samples contained 2.5% NaCl, and the higher salt level apparently enhanced water binding and led to a more flexible cheese. Shear stress and shear strain decreased with storage only in the 1.0% NaCl cheeses, which indicated a weakening of its structure with time. Shear rigidity, an indicator of rubbery (low values) and tough (high values) texture, was relatively constant during storage. The RM cheeses, with their low moisture, were more rubbery than the commercial PM cheeses.

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Table III. Results of texture profile analysis and small amplitude oscillatory shear analysis of Queso Fresco in study of cheeses made from raw milk (RM) and pasteurized milk (PM), and in studies of storage temperature and NaCl content

	Hardness (N)	*Cohesiveness*	*Elastic modulus (kPa)*	*Viscous modulus (kPa)*
Commercial sample study				
Mexican RM	10.5^b	0.30^a	7.1^b	2.15^b
Mexican PM	13.1^b	0.25^b	9.1^b	3.05^b
U.S. PM	22.1^a	0.17^c	26.9^a	7.40^a
Storage temperature study				
1 wk, 4°C	13.3^a	0.17^a	11.7^a	3.19^a
8 wk, 4°C	14.1^a	0.13^a	13.6^a	3.89^a
1 wk, 10°C	12.2^a	0.19^a	13.1^a	3.50^a
8 wk, 10°C	13.1^a	0.17^a	15.4^a	4.28^a
NaCl content study				
1.0% NaCl	9.9^a	0.21^a	15.9^a	4.44^a
1.5% NaCl	10.5^a	0.17^a	16.6^a	4.79^a
2.0% NaCl	10.2^a	0.16^a	17.3^a	4.84^a

[abc]In each study, means within the same column with different subscripts are different ($P < 0.05$). Length of storage had no effect on the values in the NaCl content study; values are averages from all storage times.

Melt and Color

The melt and color properties of QF made by DFFRU and stored at 4°C were investigated. None of samples melted, which is normal for QF, even though all of the cheeses were full-fat. The colloidal $CaPO_4$ remained in the casein matrix because of the high pH levels, and the fat globules were more tightly associated with the matrix because of the milk homogenization, preventing the cheese from melting.

Significant changes in the color properties were observed upon heating (Table V). Large changes in hue were observed, as the a* values went from positive to negative upon heating. Before heating, the hue of 1 wk samples was measured at 87.2-87.8 regardless of NaCl content. After heating to 232°C for 5 min (broiling conditions) the hue was -78.1, and when heated at 130°C for 30 min (baking conditions) the range was from -84.0 to -85.3. Hue was unaffected by NaCl content or storage time. The ΔE values decreased as NaCl content increased; baked QF exhibited values of 17.3-22.0 and broiled samples ranged from 5.7 to 6.5. The top surfaces of the cheeses remained white or turned yellow upon broiling, with browning along the edges of some samples. Free whey contributed to the browning. The ΔE values generally remained stable during storage at 4°C. Table V shows that chroma was unchanged with storage time and NaCl treatment in the baked samples. The unheated and broiled samples containing 0 and 1% NaCl showed increases in chroma with time, though chroma was unchanged at higher NaCl contents. QF with elevated NaCl levels appears to have more stable color before and after cooking.

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Table IV. Results of torsion analysis of Queso Fresco in study of cheeses made from raw milk (RM) and pasteurized milk (PM), and in studies of storage temperature and NaCl content

	Shear stress (kPa)	*Shear strain*	*Shear rigidity (kPa)*
	Commercial sample study		
Mexican RM	8.0^{b}	1.11^{a}	7.8^{b}
Mexican PM	18.1^{a}	0.89^{ab}	19.7^{a}
U.S. PM	16.2^{ab}	0.66^{b}	25.0^{a}
	Storage temperature study		
1 wk at 4°C	10.0^{a}	0.71^{a}	14.5^{a}
8 wk at 4°C	10.9^{a}	0.83^{b}	13.7^{ab}
1 wk at 10°C	9.4^{a}	0.76^{ab}	12.9^{ab}
8 wk at 10°C	9.4^{a}	0.83^{b}	11.2^{b}
	NaCl content study		
1.0% NaCl, 1 d	9.7^{a}	0.87^{a}	11.5^{a}
1.0% NaCl, 8 wk	6.7^{bc}	0.75^{b}	9.1^{a}
1.5% NaCl, 1 d	9.8^{a}	0.83^{a}	12.3^{a}
1.5% NaCl, 8 wk	9.8^{a}	0.83^{a}	11.8^{a}
2.0% NaCl, 1 d	9.4^{a}	0.77^{ab}	12.3^{a}
2.0% NaCl, 8 wk	8.6^{ab}	0.73^{b}	11.7^{a}

^{abc}In each study, means within the same column with different subscripts are different ($P < 0.05$).

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Table V. Color intensity (chroma values) of Queso Fresco under various heating conditions

	No heating	*130°C for 30 min*	*232°C for 5 min*
1.0% NaCl, 1 d storage	7.6^{b}	14.1^{a}	12.1^{c}
1.0% NaCl, 8 wk storage	7.9^{a}	14.3^{a}	13.9^{a}
1.5% NaCl, 1 d storage	7.6^{b}	15.8^{a}	11.7^{c}
1.5% NaCl, 8 wk storage	7.9^{a}	15.6^{a}	12.9^{b}
2.0% NaCl, 1 d storage	7.6^{b}	15.2^{a}	11.6^{c}
2.0% NaCl, 8 wk storage	7.7^{ab}	15.6^{a}	12.3^{c}

^{abc}Means within the same column with different subscripts are different ($P < 0.05$).

Microstructure

SEM images of QF made by DFFRU and stored for 1 d and 8 wk at 4°C are shown in Figure 1. The light areas correspond to the casein matrix and the round dark areas to fat globules. No microorganisms were seen in any of the images. Semi-hard and hard cheeses with pH 5.0-5.6 normally have a fused matrix (*23*, *24*), but QF did not. Instead, fat globules 0.5-2.5 µm in diameter, surrounded by a granular and rough-surfaced matrix, were observed in all of the samples. The microstructure was consistent with a crumbly cheese and did not change appreciably with storage or varying NaCl content. Degradation of the protein matrix during storage, which is observed with ripened cheeses, would have resulted in a more open structure after 8 wk, possibly accompanied by fat globule aggregation.

Conclusions

QF may be prepared with as little as 1.0% NaCl and stored at a temperature as high as 10°C for 8 wk without seriously impacting its characteristics. In these studies, the laboratory-made cheeses were liked by a taste panel and were often indistinguishable from the commercial product. The action of spoilage microorganisms led to proteolysis, volatile compounds apparently generated from lipolysis, and lower lactose and pH levels. None of the cheeses melted despite being full-fat. The texture and microstructure were not significantly altered during storage, although some aspects of torsion and color did change. The information obtained should lead to improved quality of QF.

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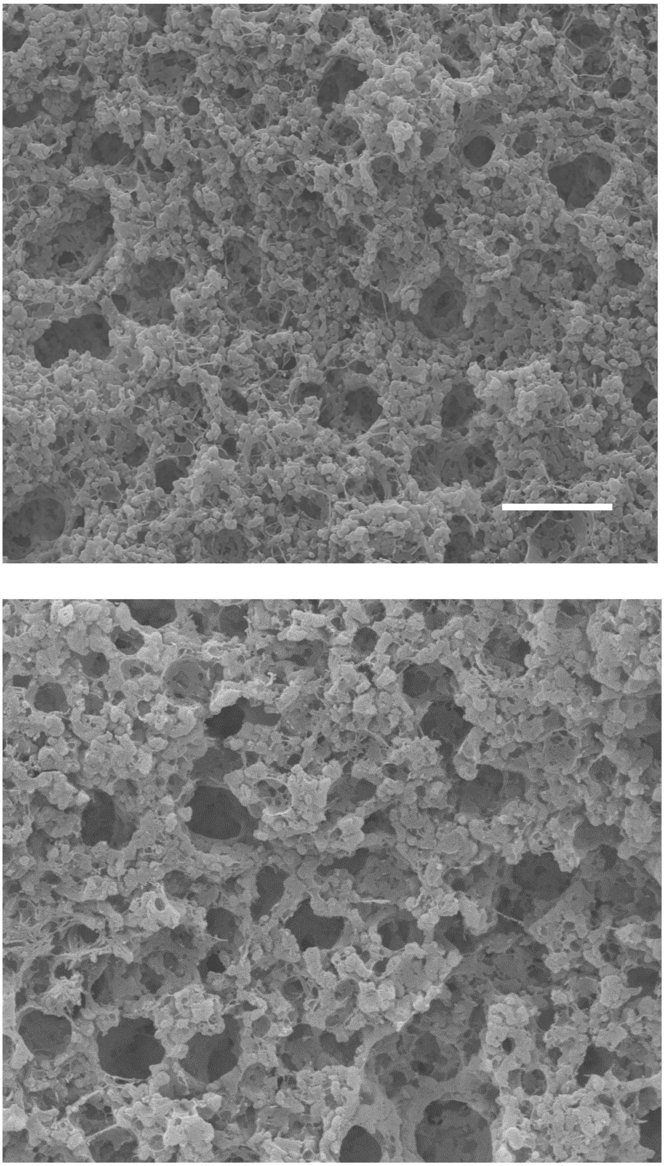

Figure 1. Scanning electron micrographs of Queso Fresco after 1 wk (top) and 8 wk of storage at 4°C. Bars at lower right correspond to 2 μm.

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Chapter 3

Comparative Analysis of a Variety of Chili Peppers: Including Components Identified in Chili Peppers for the First Time

Neil C. Da Costa,* David Agyemang, Amanda M. Bussetti, Kenneth J. Kraut, and Laurence Trinnaman

International Flavors & Fragrances Inc., 1515 Highway 36,
Union Beach, NJ 07735
***E-mail: neil.dacosta@iff.com**

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In-depth volatiles analysis was conducted on several highly pungent chili peppers, including habanero (*Capsicum chinense*), green Serrano, red chili (*Capsicum annuum*), cumari (*Capsicum praetermissum*) and red and green malagueta (*Capsicum frutescens*) varieties. Of interest amongst the many complex volatiles compositions, were several esters particularly common to chili peppers including the 4-methylpentyl analogues. In addition various capsaicinoids, macrocyclic lactones and aliphatic amides molecules are presented in this paper; some for the first time. Comparison of the compositional ratios of components in the steam distillation extracts are presented for various key components and between different liquid extraction techniques: steam distillation versus liquid/liquid extraction. In addition, for the several synthesized molecules, sensory evaluations are presented for the first time.

Introduction

The genus *Capsicum* comprises of over two hundred varieties of chili peppers for which the “fruit” varies greatly in shape, size, color, flavor and degree of pungency. There are six main species: jalapeno, Serrano, bell (*1–7*) (*Capsicum annuum*), malagueta, Tabasco (*8*, *9*), (*Capsicum frutescens*), cumari (*Capsicum praetermissum*), habanero, Scotch bonnet (*10*, *11*) (*Capsicum chinense),* aji (*Capsicum baccatum*) and rocoto, manzano (*Capsicum pubescens*). Many

In Hispanic Foods: Chemistry and Bioactive Compounds; Tunick, M., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

subspecies and hybrids stem from these. The chili pepper varieties studied and presented in this paper were habanero (*Capsicum chinense*), green Serrano, red chili (*Capsicum annuum*), cumari (*Capsicum praetermissum*) and red and green malagueta (*Capsicum frutescens*). In terms of Scoville rating units, the measure of piquancy or pungency, they can be classed as hot 10,000 to 25,000 units to ultra hot >100,000 units. Figure 1 shows the chili pepper fruits of all five varieties studied and their Scoville ratings.

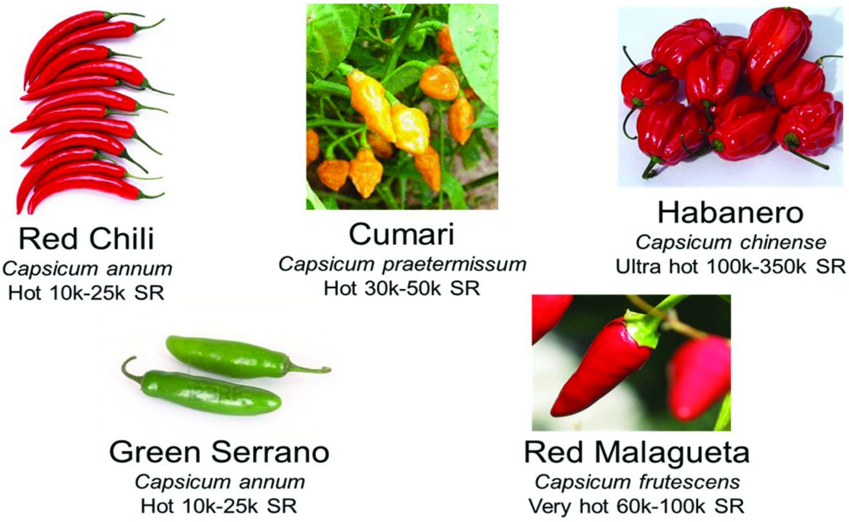
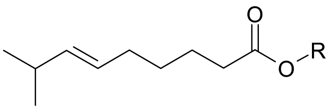
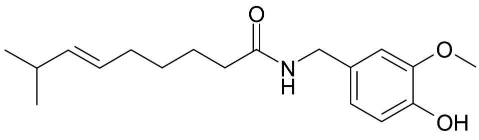

Red Chili
Capsicum annum
Hot 10k-25k SR
Cumari
Capsicum praetermissum
Hot 30k-50k SR
Habanero
Capsicum chinense
Ultra hot 100k-350k SR
Green Serrano
Capsicum annum
Hot 10k-25k SR
Red Malagueta
Capsicum frutescens
Very hot 60k-100k SR

Figure 1. Chili Pepper Varieties Analytically Studied.

Many key components and characteristic esters have been reported in chili pepper volatiles (*1*, *2*, *10*, *11*). Elmore *et al* (*12*) postulated various new esters in green Serrano chili peppers. These contained a moiety found in capsaicin and derived from 8-methyl-(*E*)-6-nonenoic acid (Fig. 2). They reported the ester derivatives where R = ethyl, 3-methylbutyl and 4-methylpentyl groups.



8-Methyl-(*E*)-6-nonenoate



Capsaicin, N-Vanillyl-8-methyl-(*E*)-6-nonenamide

Figure 2. Capsaicin and Related Esters.

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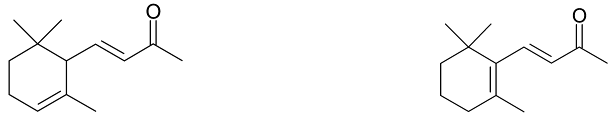
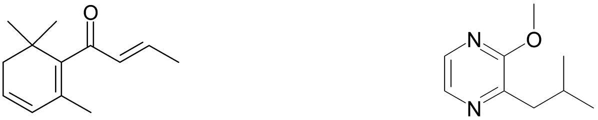
Other key, high impact, trace components reported in chili peppers are *α*-ionone, *β*-ionone, (*E*)-*β*-damascenone and the highly potent pyrazine, 2-isobutyl-3-methoxypyrazine (Galbazine); the latter having the characteristic aroma of green bell pepper (*1*, *2*, *13*). All four compounds (Fig. 3) were found to be present in trace quantities in all five chili pepper varieties studied.



α-Ionone
Floral, violet-like, raspberry

β-Ionone
Woody, berry, floral, fruity



(*E*)-*β*-Damascenone
Woody, floral, green, fruity

2-isoButyl-3-methoxypyrazine
Green bell pepper aroma

Figure 3. Key Trace Components in Chili Peppers with Aroma Descriptors.

Materials and Methods

Simultaneous Steam Distillation Extraction (SDE)

Chili peppers (200 g) were finely chopped and charged into a Likens-Nickerson apparatus (5 L vessel) with 2.5 L distilled water. The peppers were extracted by steam distillation over 3 h into methylene chloride (150 mL) (all chemicals from Sigma-Aldrich, St. Louis, MO) with internal standard, diethyl phthalate. The cooled extract was dried over anhydrous sodium sulfate, filtered and concentrated to 1 mL using a Zymark Turbovap® (Caliper Technologies, Mountain View, CA).

Liquid/Liquid Extraction (L/L)

Chili peppers (200 g) were finely chopped and placed in a 1 L beaker. Methylene chloride (400 mL) was added to cover the peppers and they were steeped overnight with occasional agitation. The solids were filtered off and the extract transferred to a separating funnel. A small quantity of water was separated off and the methylene chloride extract was dried over anhydrous sodium sulfate, filtered and concentrated to 2 mL using a Zymark Turbovap®.

Gas-Chromatography Analysis (GC)

Each steam distillate and/or liquid/liquid extract was analyzed using an HP6890 gas chromatograph with split/splitless injection and a flame ionization detector (FID) (Hewlett Packard, Wilmington, PA). The extract was injected onto an OV-1 capillary column (50 m x 0.32 mm i.d., 0.5 μm film thickness, Restek, Bellefonte, PA) in split (split ratio 15:1) and splitless modes. Carrier gas was hydrogen with a flow rate of 1.0 mL/min. The injection port temperature was 250°C and the detector temperature 320°C. The column temperature program was from 40°C to 270°C at a rate of 2°C/min and a holding time at 270°C of 10 min. The extract was also injected into a HP6890 gas chromatograph with split/splitless injection and a flame ionization detector (FID) fitted with a carbowax capillary column (50 m x 0.32 mm i.d., 0.3 μm film thickness, Restek) using the same injection and detection parameters. GC oven temperature program was with an initial temperature of 40°C held for 10 min, ramped at 2°C/min to a final temperature of 220°C and held for 20 min. To aid in the detection of sulfur containing compounds, the extract was analyzed by HP6890 gas chromatograph equipped with an Antek chemiluminescence detector (Hewlett Packard, Wilmington, PA). The column was an OV-1 capillary column and the analysis was conducted in splitless mode. Injection and detection temperatures as well as temperature program were as described for the GC OV-1. All data was collected and stored by using HP ChemStation software (Hewlett Packard, Wilmington, PA).

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Gas Chromatography-Mass Spectrometry Analysis (GC-MS)

Identification of components in the extracts was conducted by mass spectrometry. The extracts were injected onto an HP6890 GC. The chromatographic conditions for the OV-1 column were the same as described for GC analysis. The end of the GC capillary column was inserted directly into the ion source of the mass spectrometer via a heated transfer line maintained at 280°C. The mass spectrometer was an Autospec high resolution, double-focusing, magnetic sector instrument (Micromass, Manchester UK). The mass spectrometer was operated in the electron ionization mode (EI), scanning from m/z 450 to m/z 33 @ 0.3 s per decade. For analysis on the carbowax phase (50 m x 0.32 mm i.d., 0.3 μm film thickness carbowax capillary column), the sample was introduced via an HP5890 GC into a Micromass Autospec mass spectrometer. GC oven conditions were the same as outlined above. Spectra obtained from both phases were analyzed on the MassLib data system (MPI, Mulheim/Ruhr, Germany) using an IFF in-house library and the commercial Wiley 8, NIST 98 and other libraries. The identification of flavor components was confirmed by interpretation of MS data and by GC linear retention indices based on calibration with alkanes.

Gas Chromatography-Olfactometry Analysis (GC-O) and Chemical Evaluation

For the gas chromatography-olfactometry session, the extracts were injected into an HP6890 gas chromatograph equipped with flame ionization detector (FID) and odor port Model ODP-2 (Gerstel, Inc., Baltimore, MD). The FID:odor port split was 1:6. The chromatographic conditions were the same as described previously. Three trained panelists smelled through each of the five extracts, twice and recorded their aroma descriptors. From these it was possible to detect subtle aroma differences between the samples and propose new synthesis targets with potentially interesting aromas.

To evaluate the synthesized compounds a panel of typically five flavorists was used. The compounds were evaluated for aroma on smelling strips and tasted in dilutions of salt and sugar water, respectively to try and give their optimum performance for flavor use. The panels used standardized flavor descriptors to record their evaluations.

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Results and Discussion

Figure 4 shows the total ion chromatographic profile differences between two extraction techniques for the red chili pepper variety. The liquid/liquid extract chromatogram shows the main esters and is dominated by the high concentration capsaicinoids and other higher molecular weight components. This makes it harder to detect and identify low concentration components in the baseline. In contrast the steam distillation extract gives great detail of trace components. The lack of high boiling components gives a cleaner extract and in this case lacks the higher concentration capsaicinoids. Thus these extraction techniques complement each other for quantification and quantitation purposes.

Figure 5 shows a portion of the liquid/liquid chromatogram of red chilies and the homologous series of capsaicin related compounds plus their Scoville unit ratings, where known (*14–21*). Capsaicin and dihydrocapsaicin dominate the chromatogram and are the most pungent components detected so far in chili peppers. Note the lesser known capsaicinoids at either end of the series.

Figure 6 shows another portion of the liquid/liquid chromatogram where a homologous series of aliphatic acetamides are newly reported in red chili peppers as far as the authors are aware. Not the whole series was synthesized, but could be postulated from mass spectral interpretation and predicted linear retention times.

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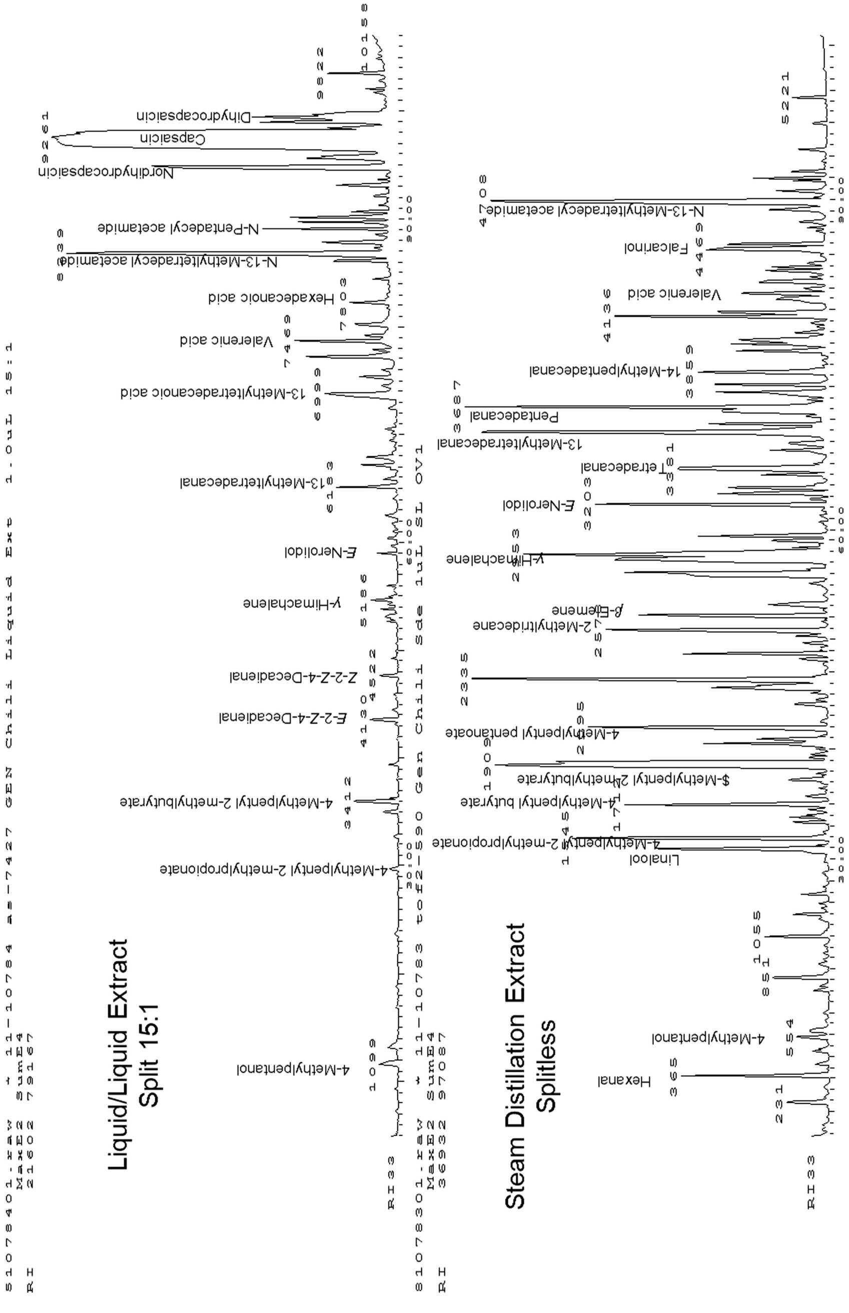

Figure 4. Liquid/liquid extract v Steam Distillate of Red Chilis.

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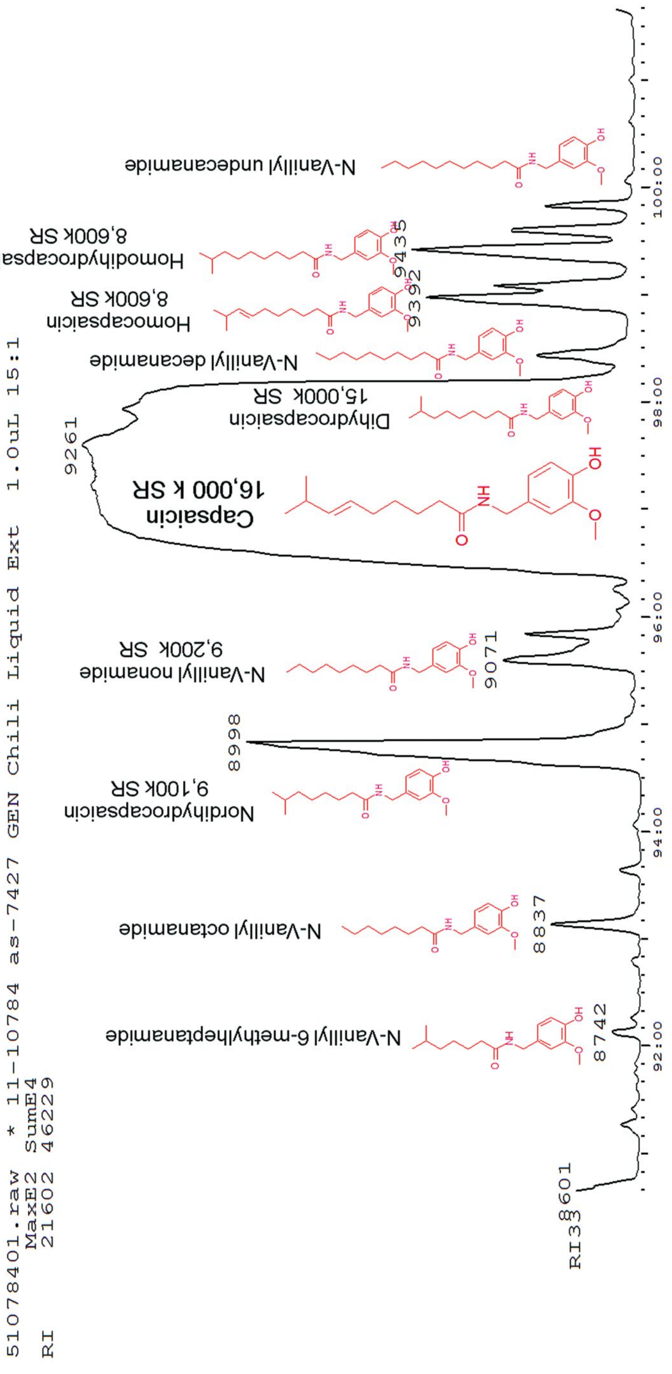

Figure 5. Capsaicinoids in Red Chili Peppers.

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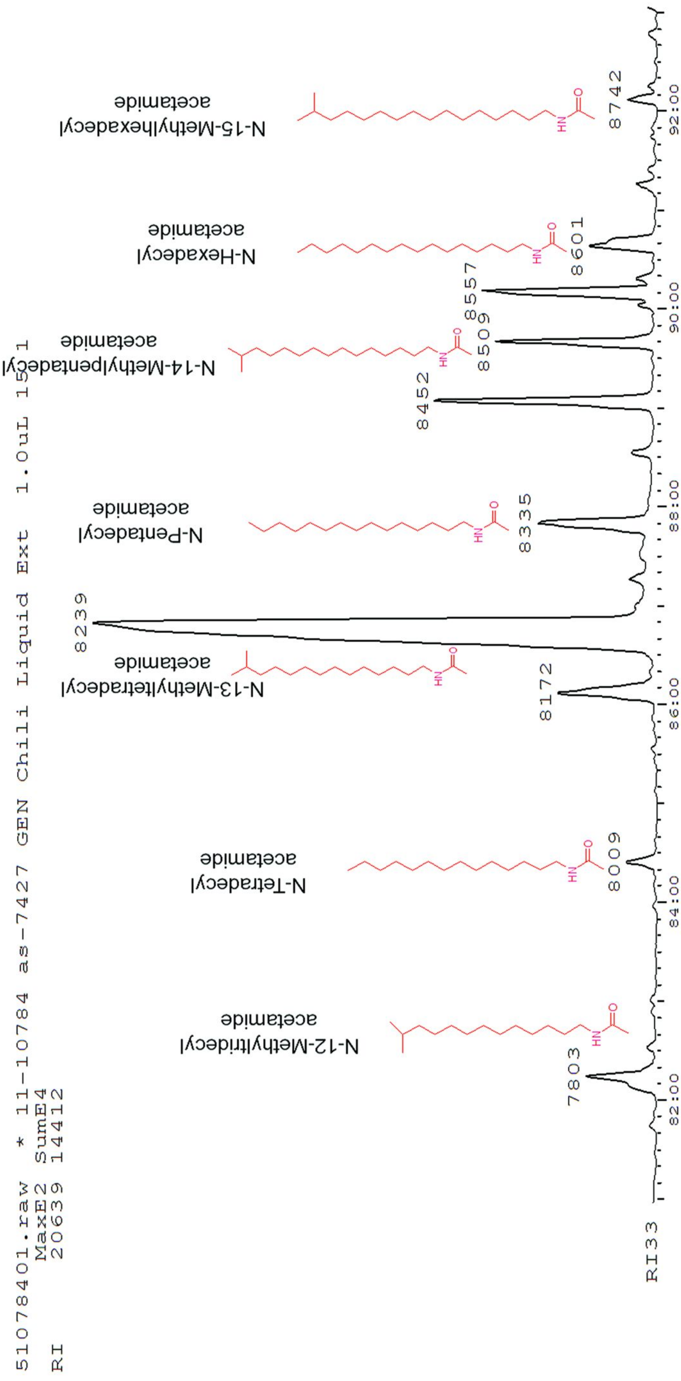
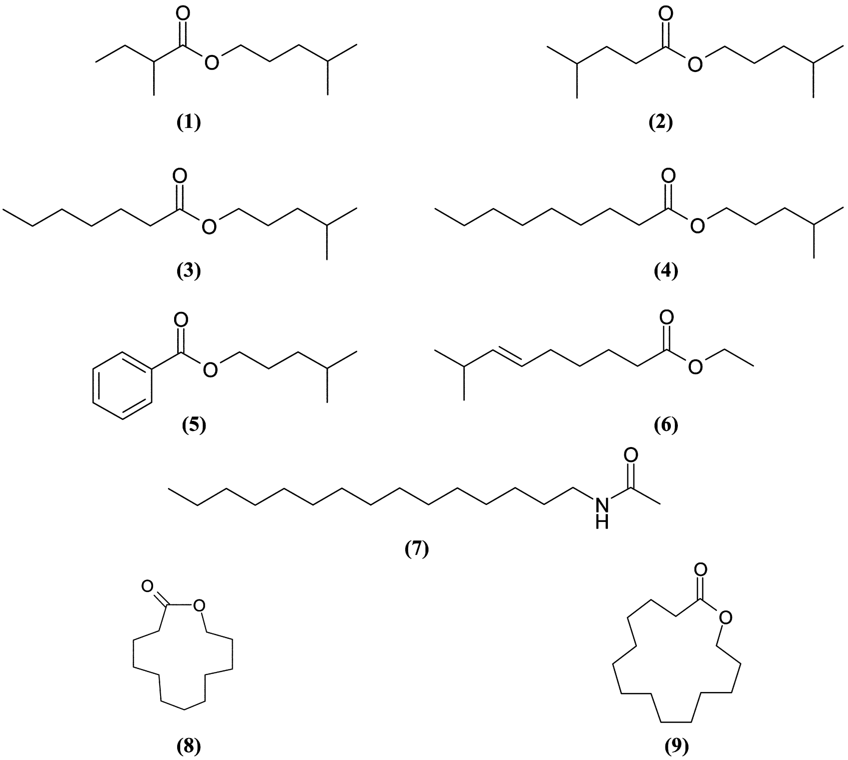


Figure 6. Aliphatic Amides in Red Chili Peppers.

Synthesis, Analytical, and Sensory

The following compounds (Fig. 7) were synthesized, based on analytical structural elucidation and interest by GC-olfactometry. Synthesis, analytical and sensory data are reported for all nine compounds.



(1) **(2)** **(3)** **(4)** **(5)** **(6)** **(7)** **(8)** **(9)**

Figure 7. Synthesized Chili Pepper Compounds.

A mixture of 4-methylpentanol (0.791 mol), aliphatic acid (0.659 mol) and sulfuric acid (0.033 mol) was heated at reflux for 1 h. The reaction mixture was cooled to room temperature, water added and stirred for 15 min. The organic layer was washed with saturated sodium bicarbonate solution followed by brine. The crude material was distilled under reduced pressure to give the product (0.499 mol). All other esters followed this synthesis route (Fig. 8).

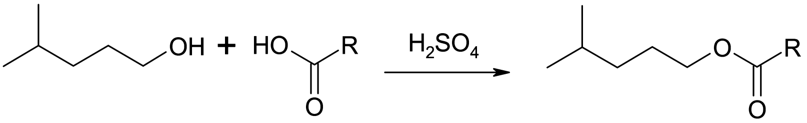


Figure 8. Ester Synthesis Route.

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4-Methylpentyl 2-methylbutyrate **(1)** (MW 186) $C_{11}H_{22}O_2$, 500-MHz 1H NMR ($CDCl_3$) 4.01-4.10 ppm (m, 2H), 2.32-2.40 ppm (m, 1H), 1.42-1.73 ppm (m, 5H), 1.20-1.26 ppm (m, 2H), 1.14 ppm (d, 3H, J=7.00 Hz), 0.91 ppm (t, 3H, J=7.45 Hz), 0.89 ppm (d, 6H, J=6.60 Hz), Flavor: fruity, creamy, waxy, savory, strawberry, apple, pear, soapy.

4-Methylpentyl 3-methylbutyrate **(2)** (MW 186) $C_{11}H_{22}O_2$, 500-MHz 1H NMR ($CDCl_3$) 4.05 ppm (t, 2H, J=6.78 Hz), 2.18 ppm (d, 2H, J=6.90 Hz), 2.06-2.15 ppm (m, 1H), 1.52-1.66 ppm (m, 3H), 1.20-1.26 ppm (m, 2H), 0.96 ppm (d, 6H, J=6.60 Hz), 0.89 ppm (d, 6H, J=6.60 Hz), Flavor: strawberry, pineapple, metallic, anisic, ripe fruit, berry, tutti frutti.

4-Methylpentyl heptanoate **(3)** (MW 214) $C_{13}H_{26}O_2$, 500-MHz 1H NMR ($CDCl_3$) 4.05 ppm (t, 2H, J=6.80 Hz), 2.29 ppm (t, 2H, J=7.55 Hz), 1.53-1.65 ppm (m, 5H), 1.25-1.35 ppm (m, 6H), 1.20-1.25 ppm (m, 2H), 0.89 ppm (d, 6H, J=6.65 Hz), 0.88 ppm (t, 3H, J=6.85 Hz), Flavor: solventy, ethereal, brown, green bean, metallic, slight pepper, cinnamyl, anise.

4-Methylpentyl nonanoate **(4)** (MW 242) $C_{15}H_{30}O_2$, 500-MHz 1H NMR ($CDCl_3$) 4.05 ppm (t, 2H, J=6.75 Hz), 2.29 ppm (t, 2H, J=7.48 Hz), 1.53-1.71 ppm (m, 2H), 1.20-1.30 ppm (m, 12H), 0.89 ppm (d, 6H, J=6.60 Hz), 0.88 ppm (t, 3H, J=7.35 Hz), Flavor: astringent, vegetative, green, slight alliaceous.

4-Methylpentyl benzoate **(5)** (MW 206) $C_{13}H_{18}O_2$, 500-MHz 1H NMR ($CDCl_3$) 8.04 ppm (d, 2H, J=7.30 Hz), 7.52 ppm (t, 1H, J=7.35 Hz), 7.41 ppm (t, 2H, J=7.63 Hz), 4.29 ppm (t, 2H, J=6.73 Hz), 1.72-1.79 ppm (m, 2H), 1.60 ppm (septet, 1H, J=6.63 Hz), 1.32 ppm (d, 2H, J=8.25 Hz, of t, J=7.20 Hz), 0.91 ppm (d, 6H, J=6.65 Hz), Flavor: vinyl, ethereal.

Ethyl 8-methyl-(*E*)-6-nonenoate **(6)** (MW 198), $C_{12}H_{22}O_2$, 500-MHz, 1H NMR ($CDCl_3$) 5.28-5.47 ppm (m, 2H), 4.08 ppm (q, 2H, J = 7.1 Hz), 2.30 ppm (t, 2H, J = 7.3 Hz), 2.24 ppm (m, 1H), 1.97 ppm (m, 2H), 1.57 ppm (m, 2H), 1.36 ppm (m, 2H), 1.24 ppm (t, 3H, J = 7.1 Hz), 0.95 ppm (d, 6H, J = 6.8 Hz). Aroma; green, thiamine, Flavor: sulfurol like, thiamine, chicken, blue cheese, fatty, waxy, cooked, green.

A mixture of pentadecan-1-amine (10.0 g, 0.044 mol) and triethylamine (4.89 g, 0.048 mol) in dichloromethane (200 mL) was cooled to -10°C. Acetyl chloride was added drop wise. After stirring for 1 h at <-10°C the reaction was cooled to room temperature. The organic mixture was washed with water followed by brine, dried over anhydrous magnesium sulfate, filtered and concentrated. The solid residue was recrystallized from hexanes, filtered and dried to give a white crystalline powder (10.4 g), see Figure 9.

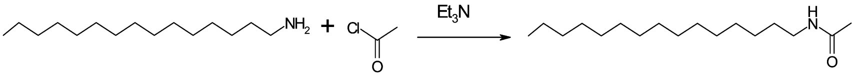

Figure 9. N-Pentadecyl acetamide Synthesis Route.

N-Pentadecyl acetamide **(7)** (MW 269) $C_{17}H_{35}O_1N_1$, 500-MHz 1H NMR ($CDCl_3$) 5.44 ppm (br. s, 1H), 3.23 ppm (q, 2H, J=6.93 Hz), 1.96 ppm (s, 3H), 1.46-1.49 ppm (m, 2H), 1.24-1.29 ppm (m, 5H), 1.25 ppm (s, 19H), 0.88 ppm (t, 3H, J=6.86 Hz), Aroma: weak seed oil, Flavor: slight throat irritation, solventy, drying, slight liver.

Dodecan-1,12-diol (4 g) was mixed with barium manganate (15.6 g, 3 equivalents) in 100 mL of acetonitrile, reaction was carried out under microwave irradiation at 150°C for 1 h. After filtration and concentration, the crude material was purified by flash chromatography (ethyl acetate, hexanes). 2.4 g of oxacyclotridecan-2-one was obtained, yield 65%, see Figure 10. (*22*). Synthesis was similar for oxacyclopentadecan-2-one.

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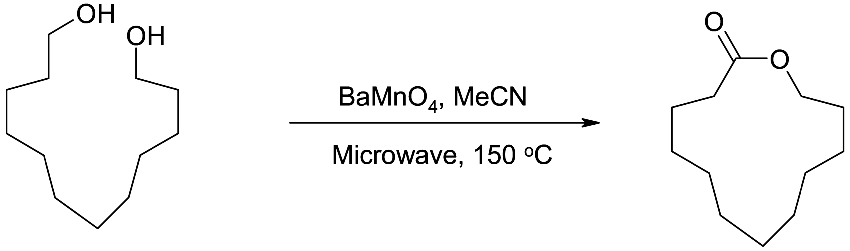

Figure 10. Oxacyclotridecan-2-one Synthesis Route.

Oxacyclotridecan-2-one **(8)** (MW 198) $C_{12}H_{22}O_2$, *m/z* M^+198(4), 180(7), 162(9), 138(18), 129(17), 110(23), 98(52), 96(43), 83(45), 69(46), 55(100), 41(85), 400-MHz 1H NMR ($CDCl_3$) 4.14-4.18 ppm (m, 2H), 2.34-2.39 ppm (m, 2H), 1.63-1.69 ppm (m, 4H), 1.28-1.45 ppm (m, 14H), Aroma: hot iron, melting wax, powerful, wet organic earth, wet soil, cedarwood like, spicy, musty, dirt, moss, borneol like, cooling, eucalyptus.

Oxacyclopentadecan-2-one **(9)** (MW 226) $C_{14}H_{26}O_2$, *m/z* M^+226(9), 208(8), 166(13), 124(20), 110(26), 98(35), 97(40), 96(44), 83(46), 82(42), 69(58), 55(100), 41(83), 400-MHz 1H NMR ($CDCl_3$) 4.10-4.16 ppm (d, 2H, J=4.48 Hz, of t, J=6.10 Hz), 2.31-2.37 ppm (d, 2H, J=4.48 Hz, of t, J=6.34 Hz), 1.62-1.70 ppm (m, 4H), 1.31-1.36 ppm (m, 18H), Aroma: floral, soapy, plastic, vinyl, heavy. musky, clean laundry, dry cleaners, ambrette seed, earthy, slight woody, pencil shavings.

Note that the aliphatic acetamides were detected in all the liquid/liquid extracts of the chili peppers. Oxacyclotridecan-2-one was detected in cumari chili peppers and oxacyclopentdecan-2-one was detected in habanero chili peppers (*11*), respectively, both at trace concentrations.

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Table I. Key Components in Five Chili Peppers

Compound	*LRI*	*Red Chili ppt*	*Green Serrano ppt*	*Red Malag. ppt*	*Green Malag. ppt*	*Cumari Chili ppt*
Linalool	1085	13.08	18.05	0.80	8.56	13.26
α-Terpineol	1177	2.81	3.61	1.02	1.59	1.92
α-Himachalene	1471	12.11	7.32	2.50	1.90	1.62
β-Himachalene	1520	6.20	1.81	1.00	0.50	1.32
γ-Himachalene	1488	46.61	18.05	21.91	19.34	7.22
Pentanal	672	1.12	1.81	1.30	0.63	0.74
Hexanal	776	29.76	3.61	2.60	1.59	14.00
(*E*)-2-Hexenal	824	1.46	9.03	3.10	1.59	5.45
(*E,Z*)-2,4-Decadienal	1246	1.78	3.61	1.70	0.63	0.74
(*E,E*)-2,4-Decadienal	1291	9.18	3.61	2.20	1.59	34.47
13-Methyltetradecanal	1678	46.68	5.42	7.40	2.22	3.98
Hexadecanal	1823	13.11	1.81	2.80	0.50	1.62
Benzaldehyde	931	4.24	3.61	0.10	0.32	0.29
2-Phenyl-2-propenal	1140	2.15	0.01	0.70	0.95	1.47
2-Amylfuran	978	3.51	9.03	1.10	0.63	3.39
α-Ionone	1410	4.16	0.01	0.03	1.90	0.03
β-Ionone	1467	19.16	5.42	6.60	3.80	0.74
Amyl alcohol	755	0.65	3.61	1.10	1.59	3.24
Hexanol	854	2.43	25.27	4.60	22.10	56.28
Hexyl 3-methylbutyrate	1225	0.53	1.81	13.91	8.56	30.49
Hexyl 2-methylbutyrate	1221	2.84	0.01	14.91	8.24	75.72
(*Z*)-3-Hexenyl 3-methylbutyrate	1218	0.84	0.02	6.60	9.83	6.33
2-Methylhexadecane	1614	9.32	3.61	1.90	3.49	1.33
2-Methylpentadecane	1518	6.26	14.44	1.50	2.22	4.71

LRI = Linear retention index on an OV-1 non-polar column.

The concentrations of key components in the various chili pepper steam distillation extractions are reported in Table I. They show that most are similar in composition. However the Serrano chili pepper extract contained many compounds related to the 6-methyl-(*E*)-8-nonenoate analogue as previously reported (*4*, *12*). Also of interest are the alpha-, beta- and gamma- himachalene sesquiterpenes which appear to be fairly characteristic of the chili peppers, beta-ionone which is present at higher concentrations than alpha-ionone and long chain aliphatic aldehydes which are also prominent.

Table II shows the concentrations of key 4-methylpentyl esters amongst the five studied chili peppers. Again serrano chili pepper is lower than the rest due to the presence of other esters.

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Table II. Key 4-Methylpentyl esters in Five Chili Peppers

4-Methylpentyl compound	*LRI*	*Red Chili ppt*	*Green Serrano ppt*	*Red Malag. ppt*	*Green Malag. ppt*	*Cumari Chili ppt*
4-Methylpentanol	820	3.04	18.05	37.72	40.27	49.35
4-Methylpentyl acetate	968	0.01	0.01	1.20	0.63	1.03
4-Methylpentyl 2-methylpropionate	1119	14.98	1.81	23.31	13.95	58.78
4-Methylpentyl butyrate	1159	8.35	0.03	1.10	0.63	0.74
4-Methylpentyl 2-methylbutyrate **(1)**	1208	13.32	1.81	125.85	39.63	46.26
4-Methylpentyl 3-methylbutyrate **(2)**	1211	70.28	9.03	97.44	31.07	100.03
4-Methylpentyl pentanoate	1260	17.16	0.01	13.51	1.90	0.06
4-Methylpentyl hexanoate	1353	2.07	0.01	19.71	6.34	3.83
4-Methylpentyl decanoate	1757	0.14	0.01	3.70	0.02	1.47
4-Methylpentyl benzoate **(5)**	1534	0.20	0.03	2.00	0.63	0.05

LRI = Linear retention index on an OV-1 non-polar column.

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Table III. 4-Methylpentyl esters, Aliphatic Acetamides & Capsaicinoids

Compound	*LRI OV1*	*LRI Cbwx*	*Mass Spectral data m/z (% intensity)*
4-Methylpentyl acetate	968	971	M^+144(0), 101(1), 84 (12), 56(28), 43(100), 42 (8), 41(12), 29(9), 27(10)
4-Methylpentyl propionate	1065	1064	M^+158(0), 115(5), 84(15), 75(40), 69(28), 57(100), 56(70), 43(66), 41(42), 42(18)
4-Methylpentyl 2-methylpropionate	1119	1095	M^+172(0), 139(1), 129(5), 89(47), 84(30), 69(23), 56(43), 43(100), 41(32)
4-Methylpentyl butyrate	1159	1136	M^+172(0), 144(1), 129(9), 89(90), 84(63), 71(83), 69(42), 56(82), 43(100), 41(48)
4-Methylpentyl 2-methylbutyrate **(1)**	1208	1149	M^+186(0), 143(6), 116(5), 103(80), 85(100), 69(40), 60(66), 57(65), 56(87), 43(85), 41(61)
4-Methylpentyl 3-methylbutyrate **(2)**	1211	1170	M^+186(0), 158(2), 143(5), 103(100), 85(69), 84(77), 69(25), 57(93), 56(66), 43(71), 41(52)
4-Methylpentyl pentanoate	1260	1231	M^+186(0), 143(10), 117(10), 103(92), 85(97), 84(68), 69(38), 57(68), 56(100), 43(94), 41(58)
4-Methylpentyl 4-methylpentanoate	1318	1290	M^+200(1), 157(8), 117(61), 99(45), 85(33), 4(60), 81(25), 69(40), 56(78), 55(28), 43(100), 41(48)
4-Methylpentyl hexanoate	1353	1328	M^+200(0), 171(1), 157(5), 117(90), 99(65), 84(71), 81(29), 69(28), 61(56), 55(40), 43(100), 41(45)
4-Methylpentyl heptanoate **(3)**	1458	1448	M^+214(1), 131(79), 113(48), 85(44), 84(80), 69(33), 57(21), 56(85), 43(100), 41(43)
4-Methylpentyl octanoate	1559	1557	M^+228(1), 185(2), 145(76), 127(30), 109(35), 84(86), 61(30), 57(45), 56(60), 55(40), 43(100), 41(45)
4-Methylpentyl nonanoate **(4)**	1661	1635	M^+242(1), 199(4), 159(53), 141(25), 84(100), 69(28), 57(30), 56(69), 55(25), 43(68), 41(31)
4-Methylpentyl decanoate	1757	1747	M^+256(1), 213(5), 173(63), 155(20), 129(19), 84(77), 61(25), 57(41), 56(56), 55(50), 43(100), 41(53)
4-Methylpentyl benzoate **(5)**	1137	1756	M^+206(1), 163(3), 123(99), 122(10), 105(100), 84(33), 77(45), 69(12), 56(30), 51(12), 43(11), 41(12)

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Table III. (Continued). 4-Methylpentyl esters, Aliphatic Acetamides & Capsaicinoids

Compound	*LRI OV1*	*LRI Cbwx*	*Mass Spectral data m/z (% intensity)*
4-Methylpentyl phenylacetate	1193	1836	M^+220(1), 136(43), 129(22), 91(100), 84(12), 83(10), 69(8), 65(17), 57(15), 56(9), 43(92), 41(23)
Ethyl 8-methyl-(*E*)-6-nonenoate **(6)**	1340	1607	M^+198(6), 152(28), 137(30), 88(39), 81(36), 69(97), 67(44), 56(38), 55(96), 43(44), 41(100), 39(36)
Oxacyclotridecan-2-one **(8)**	1537	1681	M^+198(4), 180(6), 162(8), 151(6), 138(18), 129(17), 110(22), 98(55), 96(44), 83(46), 69(46), 55(100), 41(86)
Oxacyclopentadecan-2-one **(9)**	1737	1875	M^+226(8), 208(7), 190(4), 166(14), 151(6), 138(13), 124(20), 110(26), 96(44), 83(47), 69(58), 55(100), 41(83)
N-(12-Methyltridecyl) acetamide	2037	2457	M^+255(46), 240(18), 212(25), 130(25), 114(35), 100(38), 86(45), 73(100), 72(75), 60(43), 55(37), 43(78), 41(44)
N-Tetradecyl acetamide	2071	2489	M^+255(33), 240(10), 212(10), 128(15), 114(34), 100(34), 86(40), 73(100), 72(68), 60(33), 55(28), 43(84), 41(35)
N-(13-Methyltetradecyl) acetamide	2139	2558	M^+269(53), 254(20), 226(29), 128(20), 114(42), 100(41), 86(48), 73(100), 72(80), 60(41), 55(35), 43(95), 41(40)
N-Pentadecyl acetamide **(7)**	2177	2590	M^+269(48), 254(12), 226(12), 128(15), 114(39), 100(37), 86(41), 73(100), 72(65), 60(35), 55(30), 43(80), 41(35)
N-(14-Methylpentadecyl) acetamide	2240	------	M^+283(54), 268(20), 240(28), 114(42), 100(40), 86(43), 73(100), 72(68), 60(35), 55(30), 43(85), 41(33)
N-Hexadecyl acetamide	2277	------	M^+283(35), 268(9), 240(9), 128(18), 114(37), 100(35), 86(42), 73(100), 72(65), 60(39), 55(37), 43(87), 41(40)
N-(15-Methylhexadecyl) acetamide	2340	------	M^+297(50), 282(20), 254(32), 137(30), 114(40), 100(38), 86(43), 73(100), 72(65), 60(38), 55(38), 43(80), 41(40)

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Table III. (Continued). 4-Methylpentyl esters, Aliphatic Acetamides & Capsaicinoids

Compound	*LRI OV1*	*LRI Cbwx*	*Mass Spectral data m/z (% intensity)*
N-Vanillyl 6-methylheptanamide	2333	------	M^+279(29), 195(14), 152(12), 151(10), 137(100), 122(9), 57(7), 55(7), 43(12), 41(8)
N-Vanillyl octanamide	2383	------	M^+279(28), 195(15), 152(13), 151(10), 137(100), 122(8), 57(9), 55(7), 43(9), 41(9)
Nordihydrocapsaicin	2434	------	M^+293(40), 195(19), 152(13), 151(12), 137(100), 122(10), 57(7), 55(8), 43(12), 41(9)
N-Vanillyl nonanamide	2484	------	M^+293(30), 195(14), 152(13), 151(12), 137(100), 122(10), 55(6), 53(7), 43(10), 41(8)
Capsaicin	2509	------	M^+305(19), 195(7), 152(13), 151(9), 137(100), 122(8), 69(7), 55(10), 41(12)
Dihydrocapsaicin	2535	------	M^+307(28), 195(16), 152(10), 151(10), 137(100), 122(8), 69(7), 55(10), 41(12)
N-Vanillyl decanamide	2585	------	M^+307(5), 195(8), 152(10), 151(7), 137(100), 138(10), 122(7), 55(6), 43(5), 41(4)
Homocapsaicin	2620	------	M^+319(15), 195(8), 152(11), 151(5), 122(7), 138(10), 137(100), 122(7), 94(5), 55(10), 41(10)
Homodihydrocapsaicin	2634	------	M^+321(25), 195(19), 152(12), 151(11), 138(13), 137(100), 122(8), 94(4), 55(5), 43(13), 41(8)
N-Vanillyl undecanamide	2684	------	M^+321(23), 195(15), 152(11), 151(10), 138(11), 137(100), 122(7), 55(7), 43(11), 41(8)

LRI = Linear retention index on an OV-1 non-polar & Cbwx polar column.

Table III shows the mass spectral and LRI data for the complete series of 4-methylpentyl esters, several aliphatic acetamides and capsaicinoids. The latter groups could only be detected in the liquid/liquid extract of the chili peppers and not the steam distillate. Note that all chili peppers studied were extracted by steam distillation but not all by liquid/liquid extraction. Due to their higher molecular weights some of the aliphatic acetamides and most of the capsaicinioids did not elute on a polar phase, thus only their non-polar LRI values are reported.

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Conclusions

Analyses of five chili pepper varieties were presented in this paper. However this was part of a greater chili pepper analytical study resulting in over eight hundred volatile and semi-volatile components being detected. This paper gives analytical data on the complete series of 4-methylpentyl esters, many aliphatic acetamides and capsaicinoids. In addition, sensory data is reported on several compounds for the first time as far as the authors are aware. Several of these esters are key to the unique chili pepper flavors. The aliphatic amides may contribute more in mouthfeel or display slight sensate properties. The cyclic lactones also appear to be flavor components of interest. They are possibly derived by intra-molecular cyclization of some of the long-chain unsaturated aliphatics present.

More recent research on chili peppers has been focused on their non-volatile composition, but there is still plenty of scope for new volatile molecule discovery. Amongst the hundreds of chili pepper varieties and hybrids there must be more characteristic components to be discovered.

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Chapter 4

Bioactive Compounds in Peppers and Their Antioxidant Potential

G. K. Jayaprakasha,*,[1] Haejin Bae,[1] Kevin Crosby,[1] John L. Jifon,[1,2] and Bhimanagouda S. Patil[1]

[1]Vegetable and Fruit Improvement Center, Department of Horticultural Sciences, Texas A&M University, 1500 Research Parkway A120, College Station, TX 77845-2119
[2]Texas A&M AgriLife Research, 2415 E. Hwy 83, Weslaco, TX 78596
***E-mail: gkjp@tamu.edu**

Substantial recent research has focused on foods containing antioxidant bioactive compounds. Peppers (*Capsicum annuum*) are a good source of antioxidants and nutrients, as well as bioactive compounds such as flavonoids, phenolic acids, carotenoids and vitamins C, E, A and are also rich in natural colors and aromas. The key bioactive compounds found in peppers including flavonoids, capsaicinoids and capsinoids have been linked to biochemical and pharmacological effects including anti-oxidation and anti-inflammation activities. Capsaicinoids provide the pungent sensation in hot peppers whereas capsinoids are non-pungent compounds present in sweet peppers. Capsinoids have been reported to have anti-inflammatory activity as well as to promote energy consumption and to suppress fat accumulation increase body temperature in humans. The activities of capsinoids, and their lack of pungency, make them attractive for potential applications in food and pharmacology. Other major bioactive compounds of peppers include ascorbic acid, carotenoids, and other antioxidants. The culinary properties and biological effects of bioactive compounds make them extremely important not only for nutrition, but also as pharmacological substrates.

In Hispanic Foods: Chemistry and Bioactive Compounds; Tunick, M., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

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Introduction

In recent years, awareness of the benefits of functional foods and interest in the discovery of natural bioactive compounds has risen substantially (*1–4*). Numerous plant secondary compounds, with demonstrated or proposed bioactivities have been described in many foods (*5–7*). For example, peppers are rich in bioactive compounds and are one of the most valuable vegetable crops; peppers are also widely consumed and consumption of peppers has increased by 18% from 2002 to 2006 (*8*). Peppers belong to the *Solanaceae* family and are grown as a perennial shrub in warm climatic zones of the world. The *Solanaceae* family includes about 90 genera and 2000 species. There are five recognized cultivated species of *Capsicum*, the dominant globally is *Capsicum annum*. The other cultivated commercial species are *C. baccatum, C. chinense, C. frutescens,* and *C. pubescens* (*9*). Although peppers are largely used as condiments, or constituents of dishes such as salads, they are also a good source of most essential nutrients such as flavonoids, phenolic acids, carotenoids (β-carotene, capxanthin, zeaxanthin) and vitamins as C, E, A and are also rich in natural colors and aromas (*10*). The major pungent components in Capsicum peppers are capsaicin [(*E*)-*N*-(4-hydroxy-3-methoxybenzyl)-8-methyl-6-nonenamide] and dihydrocapsaicin (*11*, *12*). Intake of these compounds in food can be an important health-protecting factor if they are taken daily in adequate amounts (*13*). Peppers are also, good source of provitamin A and oxygenated carotenoids that are important for the prevention of macular degeneration and cataracts (*14*), and phytochemicals such as flavonoids, which may reduce the risk of degenerative disease (*15*).

Reactive oxygen and cellular antioxidants are also important during fruit maturation and for maintenance of food quality. During maturation, pepper fruits undergo a transformation in color, aroma and texture; production of reactive oxygen species (ROS) plays an important role in maturation, including in the biosynthesis of carotenoids and in the transformation of chloroplasts to chromoplasts (*16*, *17*). Later, during fruit senescence, ROS are also released, cellular structures and enzymes are degraded and lipid peroxidation increases (*18*). Lipid peroxidation in foods is one of the main causes of deterioration of quality, leading to the appearance of unpleasant flavors and disagreeable scents and the destruction of vitamins. The interaction between ROS and proteins is quite complex, and the formation of carbonyl groups is considered, as an irreversible modification, to be a valuable marker of oxidative stress (*19*). In this context, the function of the cellular antioxidant system is to prevent the offset of chain oxidations and removing ROS is vital. The enzymatic system of defense includes superoxide dismutase, catalase, peroxidases and the ascorbate glutathione cycle enzymes (*20*). In the last 10 years, several reports have provided evidence for the involvement of antioxidants in fruit physiology, including a response at the level of mitochondria, peroxisomes and chloroplasts during pepper ripening and during fruit storage at 20 °C (*18*, *21*).

Non-enzymatic antioxidants include both polyphenols and non-phenolic compounds such as ascorbic acid, and carotenoids, which are important in vegetables (*22*). The human organism cannot synthesize many of these protective

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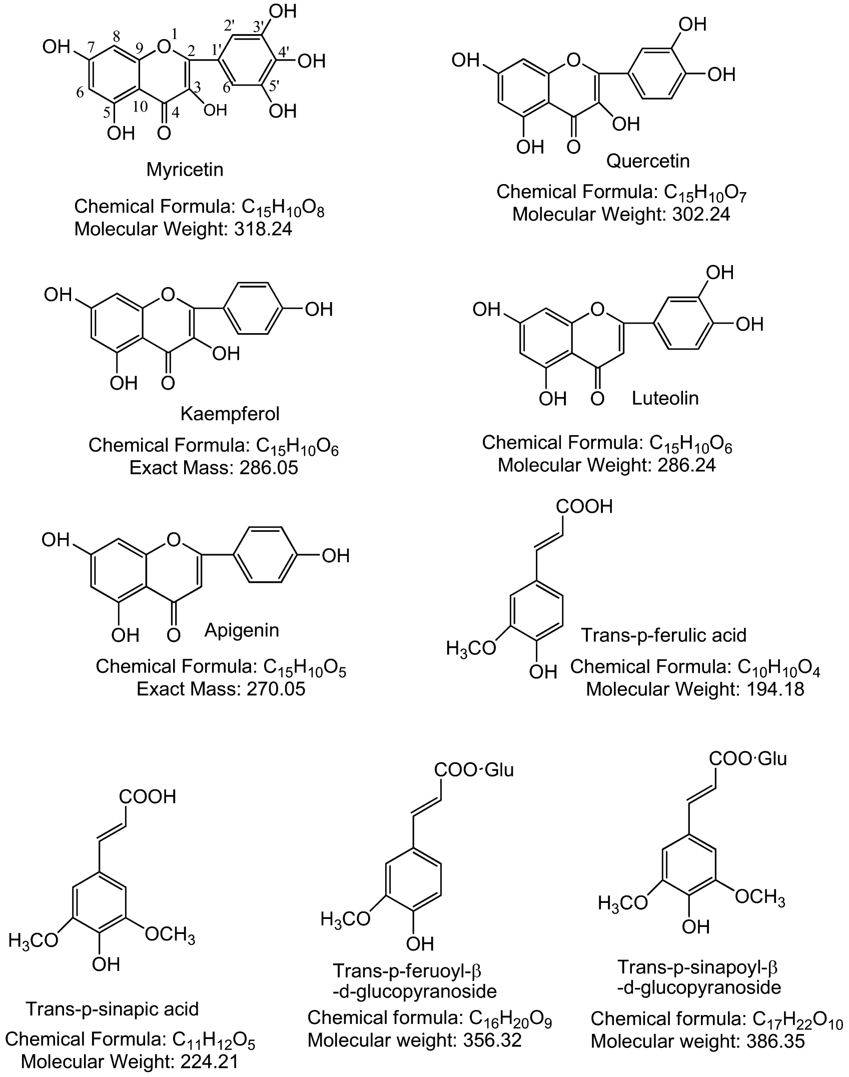
chemical substances, and they can only be obtained from foods. Polyphenols are widely distributed in plants and contribute to their color and flavor; two major classes of polyphenols, flavonoids and phenolic acids, protect the organism from the damage produced by oxidative agents. Levels of polyphenols are a good indication of the antioxidant capacity of peppers (*9*), and numerous epidemiological studies indicate a possible association between their uptake and reduction of the risk of coronary disorders and cancer (*6*).



Myricetin
Chemical Formula: $C_{15}H_{10}O_8$
Molecular Weight: 318.24

Quercetin
Chemical Formula: $C_{15}H_{10}O_7$
Molecular Weight: 302.24

Kaempferol
Chemical Formula: $C_{15}H_{10}O_6$
Exact Mass: 286.05

Luteolin
Chemical Formula: $C_{15}H_{10}O_6$
Molecular Weight: 286.24

Apigenin
Chemical Formula: $C_{15}H_{10}O_5$
Exact Mass: 270.05

Trans-p-ferulic acid
Chemical Formula: $C_{10}H_{10}O_4$
Molecular Weight: 194.18

Trans-p-sinapic acid
Chemical Formula: $C_{11}H_{12}O_5$
Molecular Weight: 224.21

Trans-p-feruoyl-β
-d-glucopyranoside
Chemical formula: $C_{16}H_{20}O_9$
Molecular weight: 356.32

Trans-p-sinapoyl-β
-d-glucopyranoside
Chemical formula: $C_{17}H_{22}O_{10}$
Molecular weight: 386.35

Figure 1. Structures of flavonoids and phenolic acids found in peppers.

Polyphenols, Flavonoids, and Their Antioxidant Potential

Flavonoids and other polyphenols (Figure 1) are ubiquitous phytochemicals and some of the most abundant and important bioactive compounds present in green, sweet, and hot peppers. Many studies have focused on identifying and quantifying flavonoid levels in peppers (*23–27*). Glycosides and aglycones of myricetin, quercetin, luteolin, kaempferol, and apigenin are also found in peppers (*23*). Flavonoids show high antioxidant and anticancer activities, and this is related to the presence of the numbers of hydroxyl groups at certain position and a double bond at the C_2-C_3 position. Based on the relationship of structure and antioxidant activity, myricetin seems to be one of the most powerful flavonoid (*28–30*).

The structures of C- and O-glycosides of flavonoids in peppers have been identified based on MS–MS fragmentation and UV spectra (*31*). The four reported quercetin glycosides are: quercetin 3-O-rhamnoside, quercetin 3-O-rhamnoside-7-O-glucoside, quercetin 3-O-glucoside-7-O-rhamnoside and quercetin glycosylated with rhamnoside-glucoside attached either at the C-3 or C-7 position (*32*). Two luteolin O-glycosides, luteolin (apiosyl-acetyl)-glucoside and luteolin 7-O-(2-apiosyl)-glucoside and five luteolin C-glycosides, luteolin 6-C-hexoside, luteolin 8-C-hexoside, luteolin 6-C-pentoside-8-C-hexoside, luteolin 6-C-hexoside-8-C-pentoside and luteolin 6,8-di-C-hexoside, were found in the pericarp of pepper fruits. In addition, two apigenin C-glycosides were also identified as apigenin 6-C-pentoside-8-C-hexoside and apigenin 6, 8-di-C-hexoside (*33*).

Flavonoid levels change depending on genetic, developmental and environmental conditions. For example, the highest amounts of flavonoids are found in the red fruits of most cultivars, except cv. Cyklon (*34*). By contrast, quercetin 3-*O*-R-L-rhamnopyranoside was the main compound in the flavonoid fraction from green fruits, and the highest amounts were reported in cv. Cyklon. The levels of quercetin glycoside were high in green fruit and decreased during ripening (*34*, *35*).

Measurement of bioactive compounds is both crucial for unraveling their effects, and difficult because of their different chemical structures. For example, the antioxidant capacity of extracts made with a specific solvent does not represent all the bioactive compounds present in the pepper, because peppers have various polar and non-polar antioxidants, such as ascorbic acid, carotenoids, capsaicinoids and flavonoids. All compounds cannot be extracted using one solvent. To reveal the full antioxidant capacity of peppers, we have studied a range of extraction conditions and solvents of different polarities, to extract both polar and non-polar bioactive compounds (*36*). Our group recently measured the levels of flavonoids including quercetin, myricetin, luteolin and kaemperol and apigenin in Paprika and Habanero peppers (Figure 1) (*23*). Ethanol and methanol extraction showed the highest total phenolics and maximum radical scavenging activity (*23*). Furthermore, eight pepper cultivars include cayenne (*C. annuum* L. cv. 'CA408' and 'Mesilla'), jalapeño (*C. annuum* L. cv. 'Ixtapa'), and serrano (*C. annuum* L. cv. 'Tuxtlas') were studied to examine the antioxidant potential and active chemical constituents in various polar fractions. Five of these cultivars were commercial types (TMH, Mesilla, Ixtapa, Tuxtlas, and TMJ) and three

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were advanced breeding lines (PA137, B58, and CA408). Different levels of flavonoids such as quercetin, luteolin, kaempferol, and apigenin were found in these pepper extracts (*10*, *36*). Flavonoids were not detected in hexane extracts and the remaining four solvents extracted differential levels of flavonoids in all pepper cultivars. Figure 2 lists the levels of flavonoids in eight different pepper cultivars, as determined using extraction with five different solvents. Hydrophilic flavonoids were extracted to the maximum using methanol. The highest levels (152.2 µg/g) of flavonoids were found in methanol extract of paprika (B58). In contrast to peppers, root vegetables such as carrot, radish, burdock, and potato contain simple polyphenolic acids and their glucosides (Figure 1). Leaf vegetables such as cabbage, chive, lettuce, and spinach has flavones and flavonols mainly in the glycoside form. Among leafy vegetables, celery and parsley are classified as herb vegetables along with peppermint, sage, oregano, and thyme (*32*, *37*). They contain aglycon forms of flavones and flavonols at relatively high levels. Interestingly, peppers found to have polyphenolics, flavones and flavonols (Figure 1).

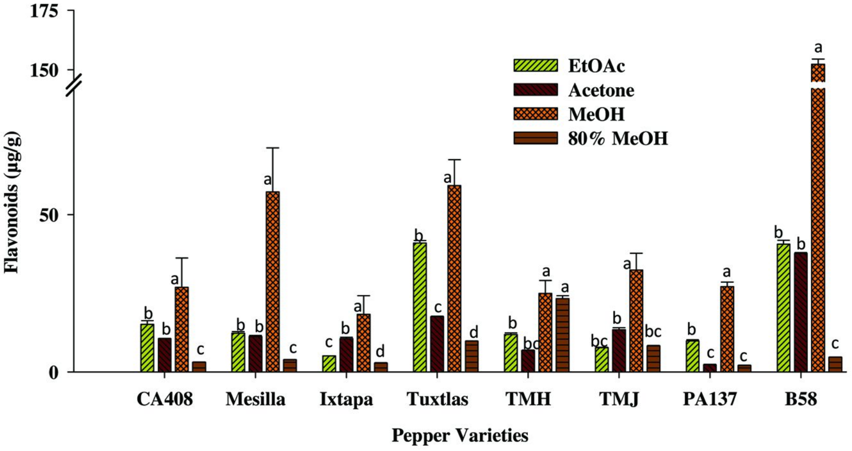

Figure 2. The levels of total flavonoids found in eight cultivars of hot and mild peppers (µg/g) determined by reversed phase HPLC (10, 37). Values are means ± standard error of triplicate analysis. The same letter within a column and a pepper cultivar is not significantly different using Tukey's test.

Levels of Capsaicinoids and Their Beneficial Properties for Human Health

In 1816, P. A. Bucholz isolated the capsaicin molecule for the first time (*38*), but the spicy properties of hot peppers have intrigued the human palate for thousands of years. Capsaicinoids are responsible for the pungent sensation in fruits of the genus *Capsicum* and the two major capsaicinoids, capsaicin and dihydrocapsaicin, and are responsible up to 90% of the total pungency of pepper fruits. Other minor capsaicinoids are nordihydrocapsaicin, homodihydrocapsaicin, homocapsaicin and nonivamide (Figure 3). The degree of pungency depends on the cultivar and environmental conditions during plant growth and fruit development/maturation Also, the levels of capsaicinoids are affected by different factors such as the developmental stage of the fruit (*39*) and the environmental growth conditions (*40–42*). Moreover, measurement of capsaicinoids also depends on extraction conditions, since peppers have a wide variety of compounds (e.g. polar and non-polar compounds), the observed functional properties such as antioxidant capacity will also depend on the assay method. Different solvents have different capacities for extracting compounds of interest from different media (*43*). The importance of optimized analytical methods for characterizing the levels and diversity of bioactive constituents in foods can, therefore, not be overemphasized. Few studies have critically assessed the importance of solvent properties in the accurate isolation and quantification of bioactive compounds in foods (*23*, *43*). The complexity of the bioactive constituents in peppers makes it difficult to isolate each compound with precision. For instance, the prevalence of pigment-fatty acid complexes in peppers makes it difficult to efficiently extract certain bioactive compounds (*44*). Thus maximum extraction requires use of a saponification step to hydrolyze these pigment-fatty acid complexes, and hence optimize extraction and simplify the quantification of bioactive compounds in pepper.

Table 1 shows the total capsaicinoid concentrations in samples from four different cultivars of hot pepper extracted with different solvents. The levels of capsaicinoid extraction were observed in the following order: hexane > EtOAC > acetone. Capsaicinoids were not found in MeOH and MeOH:water (80:20) extracts. The maximum amounts of capsaicinoids were extracted in hexane. The highest level (5072 μg/g) of capsaicinoids was found in the Ixtapa, and the lowest amount (71.5 μg/g) was found in Tuxtlas pepper (*36*). Multiple studies indicate that consuming capsaicinoids may provide health benefits; for example, these compounds are reported to inhibit iron-mediated lipid peroxidation and copper-dependent oxidation of low-density lipoprotein (*38*), an effect ascribed to their capacity to form complexes with reduced metals and act as hydrogen donors. Capsaicin also demonstrated for anti-obesity activity in an animal model (*45*). Moreover there is epidemiological evidence for an association between consumption of capsaicinoid-containing foods and lower incidence of obesity. It is widely accepted that increasing energy expenditure and reducing energy intake form the basis for management of weight (*46*, *47*). Consuming capsaicin one hour before low intensity exercise improved lipolysis and it may be a valuable supplement in the treatment of individuals with hyperlipidemia and obesity (*48*). According to animal and human studies, dietary capsaicin may be considered a functional agent that helps to prevent obesity. However, it is not ideal for controlling obesity in humans because the long-term consumption of capsaicin may be limited by its pungency. Capsaicin can also prevent the oxidation of oleic acid at cooking temperatures (*49*) as well as the formation of lipid hydroperoxides from the autoxidation of linoleic acid (*37*). Despite these intriguing findings, the use of capsaicinoids as food antioxidants is obviously limited by their strong pungency and potentially noxious properties.

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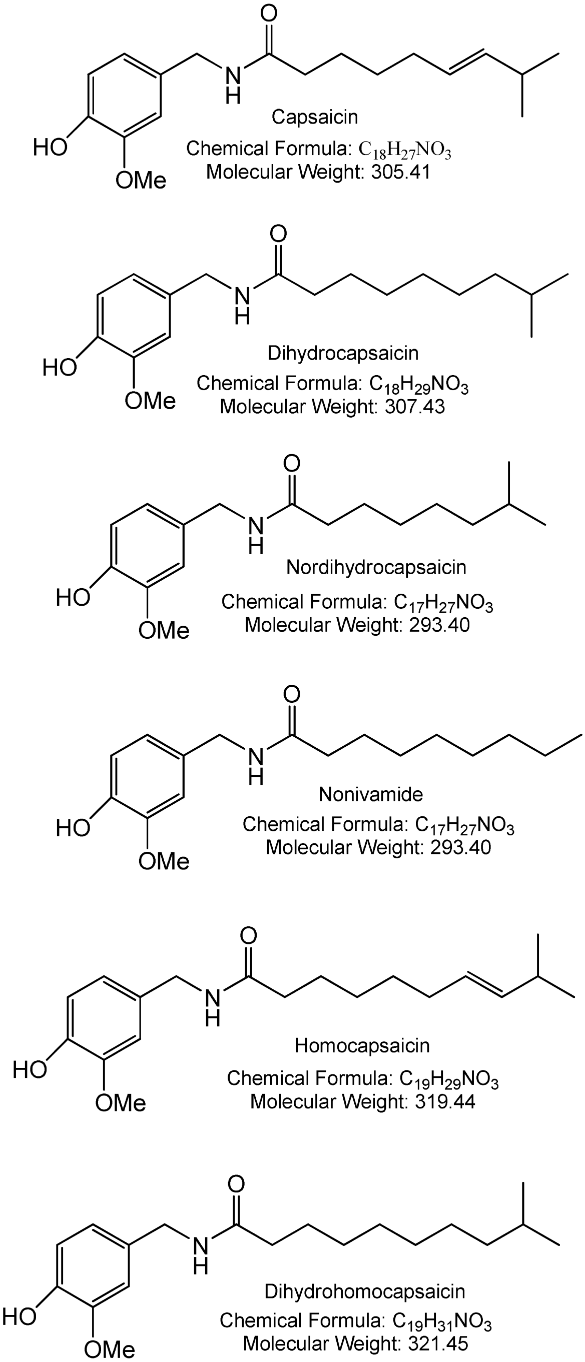

Capsaicin
Chemical Formula: $C_{18}H_{27}NO_3$
Molecular Weight: 305.41

Dihydrocapsaicin
Chemical Formula: $C_{18}H_{29}NO_3$
Molecular Weight: 307.43

Nordihydrocapsaicin
Chemical Formula: $C_{17}H_{27}NO_3$
Molecular Weight: 293.40

Nonivamide
Chemical Formula: $C_{17}H_{27}NO_3$
Molecular Weight: 293.40

Homocapsaicin
Chemical Formula: $C_{19}H_{29}NO_3$
Molecular Weight: 319.44

Dihydrohomocapsaicin
Chemical Formula: $C_{19}H_{31}NO_3$
Molecular Weight: 321.45

Figure 3. Chemical structures of capsaicinoids from hot peppers.

Table 1. Levels of capsaicinoids (µg/g) found in four hot pepper cultivars from different individual solvent and total content. Dried peppers were extracted with various solvents and quantified by HPLC (*10*, *36*)

Solvents used for extraction	*CA408*	*Mesilla*	*Ixtapa*	*Tuxtlas*
Hexane	83.8 ± 0.3	549.3 ± 12.9[a]	3511.4 ± 10.1[a]	51.9 ± 0.4[a]
EtOAC	0	18.0 ± 0.5[b]	1035.3 ± 26.8[b]	11.4 ± 1.5[b]
Acetone	0	7.4 ± 3.2[c]	525.6 ± 42.1[c]	8.2 ± 1.0[c]
Total	83.8	574.8	5072.4	71.5

Values are means ± standard error of triplicate analysis; nd: not detected. The same letter within a column and a pepper cultivar is not significantly different using Tukey's test.

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In addition to dietary benefits, capsaicin is also applied topically for chronic pain, to reduce aches and burning feelings, the frequently reported symptoms of painful neuropathy (*50*). It is currently used in topical creams and high-dose dermal patches to relieve the pain of peripheral neuropathies such as post-herpetic neuralgia caused by shingles, and can be directly applied to abdominal skin with no side effects in animal models and humans (*51*).

Capsinoids from Sweet Peppers

Capsinoids are non-offensive and devoid of pungency, having an ester group instead of the amide moiety in capsaicinoids. Capsinoids (Figure 4) are found in the fruits of non-pungent cultivars of *Capsicum annuum* L. (CH-10 sweet) peppers at <1mg/100g fresh weight (*49*, *52*) and in *Capsicum chinense* RU 72-194 at 12mg/100g FW (*11*). These compounds have several notable biological properties including powerful antioxidant activity (*53*) and chemopreventive and antineoplastic properties (*54*). Capsinoids also are reported to promote energy consumption and suppress the accumulation of fats; they also increase body temperature in humans, and have powerful anti-inflammatory activity (*48*, *50*). No effect of single doses of 1, 3, 6 or 15 mg of encapsulated capsinoids on metabolic rate was observed in a human study (*51*). However, some studies are reported to increase metabolic rate or fat oxidation after chronic consumption of capsinoids (*55*). Thus, prolonged consumption may be necessary to affect metabolic rate in humans. These potential biological effects of the capsinoids, together with their lack of pungency, may make them extremely important, since (unlike the pungent capsaicinoids) their application in both food and pharmacological preparations is not limited by pungency.

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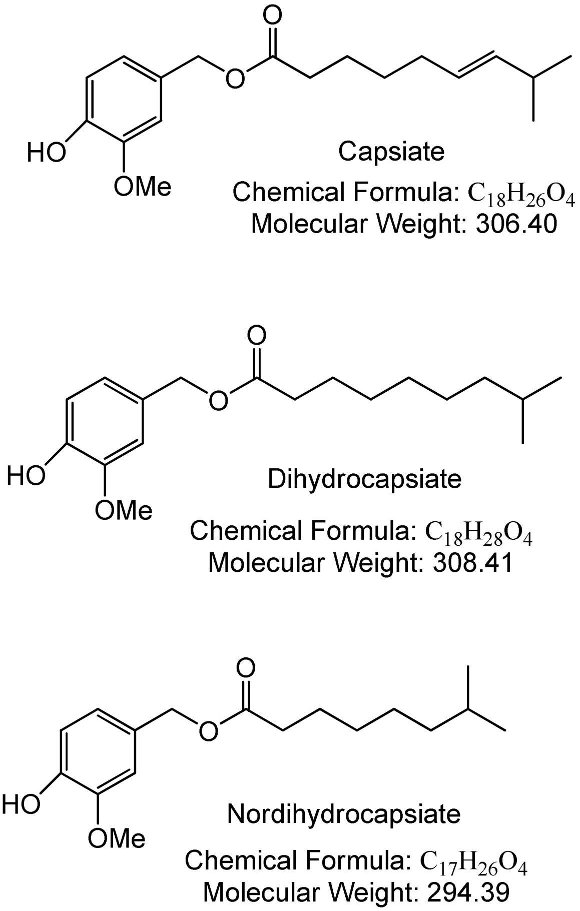

O
O
HO
OMe
Capsiate
Chemical Formula: $C_{18}H_{26}O_4$
Molecular Weight: 306.40
O
O
HO
OMe
Dihydrocapsiate
Chemical Formula: $C_{18}H_{28}O_4$
Molecular Weight: 308.41
O
O
HO
OMe
Nordihydrocapsiate
Chemical Formula: $C_{17}H_{26}O_4$
Molecular Weight: 294.39

Figure 4. Major capsinoids found in sweet peppers.

Ascorbic Acid, Carotenoids, and Antioxidant Activity of Peppers

Ascorbic acid, or Vitamin C, in the human diet is provided by vegetables and fruits and because of its antioxidant activity, ascorbic acid has attracted the attention of researchers, due to possible links with the prevention of some important diseases, including certain types of cancer (*7*). However, the relative amounts of non-phenolic antioxidant compounds such as ascorbic acid and carotenoids, and the basis for variation in the amounts, are still poorly understood, although it is known that these antioxidants are important in both oxidative stress and other processes. To quantify carotenoids, we have studied the efficacy of different extraction solvents (hexane, ethyl acetate, acetone, methanol, and a methanol:water mixture), as well as the levels of carotenoids in pungent (CA408, Mesilla, Ixtapa and Tuxtlas) and non-pungent pepper cultivars (TMH, TMJ,

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PA137, and B58) (Figure 5). The highest levels of carotenoids were found in extracts from hexane followed by ethyl acetate or acetone. In our pepper samples, the highest amount of carotenoids (722 μg/g) was found in hexane extracts, and the lowest amount (31.3 μg/g) was observed in acetone. It is well known that MeOH and MeOH:water (80:20) are poor solvents for extraction of carotenoids and we could not find carotenoid peaks in these extracts. Tuxtlas had the highest carotenoid content and Mesilla had the lowest among hot peppers. For mild peppers, B58 had the highest content of carotenoids (628.8 μg/g), and TMH had the lowest content (47.2 μg/g) in hexane extracts.

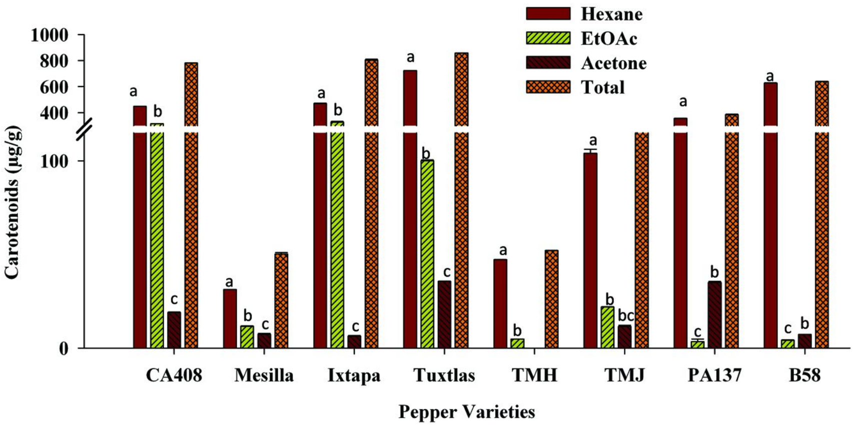

Figure 5. Carotenoids determined by HPLC in eight mature pepper cultivars extracted with different solvents (10, 36). Three solvent extract from each sample has been compared and values are expressed as means ± standard error of triplicate analysis. The same letter within a column and a pepper cultivar is not significantly different using Tukey's test.

In addition to quantification of individual compounds, antioxidant capacity can also be measured as DPPH radical scavenging activity; however, the accuracy of this measurement depends on the solvent extraction conditions. For example, DPPH radical scavenging activities of hot and mild peppers varied significantly in different extracts (Figure 6). Hexane extracts of all peppers exhibited the highest inhibition of DPPH radical scavenging activity (79.6–95.1%), but other extracts exhibited variable activity. Different pepper extracts showed variable antioxidant activities due to the selective extraction of bioactive compounds from different pepper cultivars. These results are comparable to ethanol extracts of *Capsicum annuum* var. *acuminatum* (*56*), In our results, DPPH free radical was effectively scavenged by pepper extracts from non-polar and mid polar solvents. It is possible that more carotenoids and capsaicinoids were extracted in the hexane extract (Table 1 and Figure 6). Therefore, DPPH scavenging activity was higher in hexane extract than in other extracts.

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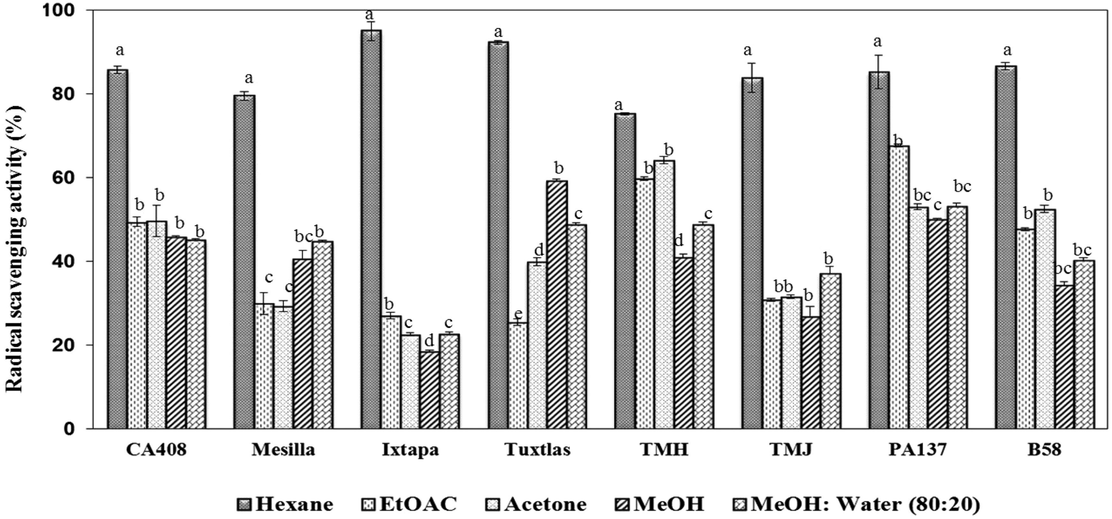

Figure 6. DPPH radical scavenging activity (%) of different solvent extracts obtained from eight pepper cultivars (10, 36). Values are means ± standard error of triplicate analysis. The same letter within a column and a pepper cultivar is not significantly different using Tukey's test.

In conclusion, peppers are a rich source of phenolic compounds, carotenoids, ascorbic acid, capsinoids and capsaicinoids, which may act to promote human health by their antioxidant and other activities. For example, the thermogenic and metabolic properties of capsinoids appear to mimic those of their more pungent sister compound capsaicin. However, more research is needed on how capsinoid/capsaicinoid ingestion affects energy expenditure in humans. Moreover, the measured concentrations of total phenolics, carotenoids, and flavonoids are highly dependent on the nature of the solvent used to extract the compounds from peppers, making high-throughput profiling of bioactive compounds challenging. Our observations demonstrated that solvents with different polarity can significantly increase the extraction efficiency of specific lipophilic or hydrophilic compounds in different peppers as well as antioxidant activity. Therefore, improved extraction and quantification methods can allow profiling of key bioactive compounds in peppers for generation of cultivars with increased health-promoting properties and can enable emerging research that will unravel the important contributions of these compounds to human health.

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Chapter 5

Ripening Research in "Maradol" Papaya: A Nutraceutical Fruit

**José Ángel Huerta-Ocampo,[1] Norma Alejandra Mancilla-Margalli,[2]
Gisela Jareth Lino-López,[3] Miguel Ángel Hernández-Velasco,[3]
Ana Paulina Barba de la Rosa,[1] and Juan Alberto Osuna-Castro*,[3]**

**[1]IPICyT, Instituto Potosino de Investigación Científica y Tecnológica,
A.C., Camino a la Presa San José No. 2055, Lomas 4ª Sección,
78216 San Luis Potosí, S.L.P., México
[2]División de Estudios de Posgrado e Investigación Instituto Tecnológico de
Tlajomulco, km 10 Carr. a San Miguel Cuyutlán Tlajomulco de Zúñiga,
Jalisco, México, CP 45650
[3]Facultad de Ciencias Biológicas y Agropecuarias, Universidad de Colima,
Autopista Colima-Manzanillo km 40, Tecomán, Colima, México CP 28100
*E-mail: josuna@ucol.mx; osuna_juan@hotmail.com**

Papaya is among the twenty top fleshy fruits produced worldwide and ranked first on nutritional scores among 38 common fruits. The fruit is mainly consumed fresh but it is also used in elaboration of drinks, jams, and as a dried and crystallized fruit candy. Several therapeutic uses have been claimed for papaya fruit. However, as climacteric fruit, it is linked to a dramatic increase in respiration and ethylene production and being susceptible to postharvest losses due to the ethylene-induced overripening and excessive softening. The inhibitor of ethylene action, 1-MCP, has been used worldwide as a safe chemical to control fruit posthaverst life and understand molecular basis of ripening. Xylanase activity was characterized and proteomics tools have shown to be a powerful tool in understanding the effects of 1-MCP on fruit ripening. New findings in papaya ripening are reported, indicating that 1-MCP is able to regulate proteins such as the TCTP and dienelactone hydrolases, in addition to SODs and GSTs, enzymes whose key roles in the activation of disease resistance mechanisms have been demonstrated.

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ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

Keywords: 1-MCP: 1-methylcyclopropene; ACC: 1-aminocyclopropane carboxylic acid; ACS: AAC synthase; ACO: ACC oxidase; AKR: aldo/keto reductase; GSTs: glutathione S-transferases; RBB-Xylan: remazol brilliant blue-xylan; pI: isoelectric point; PEs: pectinesterases; POX: guaiacol peroxidase; PR-5: pathogenesis-related family 5 of plant defense proteins; SAM synthase: S-adenosyl methionine synthase; SOD: superoxide dismutase; TCTP: translationally controlled tumor protein

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Introduction

Papaya (*Carica papaya* L.) is a major crop of tropical and subtropical regions; originally of America and widely spread after the discovery of the New World, mainly to Africa and Asia (*1*). Papaya tree takes 9-14 months seed-to-seed generation time, producing from one to three ripe fruit per week continuously throughout their reproductive life of around three years (*2*, *3*).

C. papaya is the only member of genus *Carica* and the most important economic species of the Caricaceae family. Different cultivars (or varieties) of *C. papaya* including “Solo” “Eksotika”, “Hawaiian”, “Rainbow”, “Golden”, “Sunset” and “Maradol”, among many others, could be found in different tropical regions. Originally from Cuba, “Maradol” papaya (Figure 1) is the main cultivar produced in Mexico (around 95% of the national production) and highly appreciated by its striking red-orange skin color, salmon red pulp, sweet taste and big size (*4*, *5*).

Economic Importance and Uses

Papaya is among the twenty top fleshy fruits produced worldwide (*6*), cultivated in tropical countries of America (Central and South America and the state of Hawaii), some Asian countries (mainly India and Sri Lanka), in addition to the Antilles and tropical Africa (*1*). In 2007, America was the largest producer of papaya fruit with Brazil (1,811,540 tons) and Mexico (919,425 tons) at the top; however recent statistical data of 2010 (*7*) indicate that Asia has been placed at the head with a total production of 6,178,902 tons, India being the leading producer with 4,713,800 tons, followed by Brazil and Mexico in fifth place with 616,215 tons. However the last reports of papaya exporter countries (2008) indicate that Mexico is at the top with about 90,032 tons, the US being the principal importer (*8*).

The fruit is mainly consumed fresh but it is also used in elaboration of drinks, jams, and as a dried and crystallized fruit candy (Figure 2). Pulp fruit is also used in cosmetics and employed to shampoo and facial creams. Green fruit, leaves and flowers can be cooked and consumed as a vegetable (*2*). Seeds and latex from unripe papaya contain proteolytic enzymes (papain, chymopapain, caricain and papaya proteinase IV). Papain has important industrial application; valuable to tenderize red meat, chill proofing of beer, and during external treatment of human hard tissues such as warts and scars (*2*). Alcoholic extracts of the endocarp, epicarp, roots, and seeds from ripe and unripe papaya fruit have antidiarrheic, antidysenteric and antibacterial properties (*3*). Aqueous extracts of dried papaya leaves have been shown growth suppression of different tumor cells (*2*).

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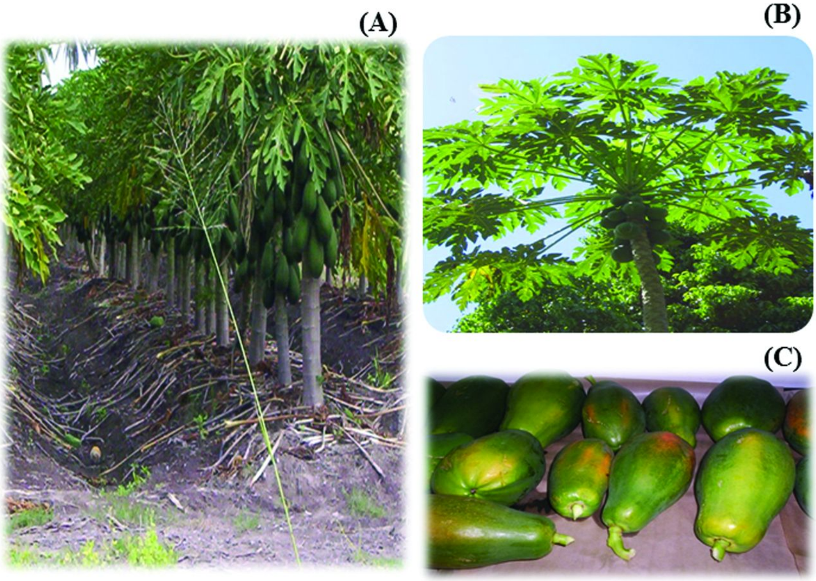

Figure 1. "Maradol" papaya trees (A and B) and fruits (C).

Papaya as Nutraceutical

Papaya is ranked first on nutritional scores among 38 common fruits (*9*). Nutritional composition of papaya fruit may vary among cultivars, maturity, and agroclimatic and postharvest conditions, but in general, 100 g of pulp could provide 0.61 g of protein, 9.8 g of carbohydrate, 1.8 g of dietary fiber, and 89% of water (*10*). Total soluble solids range from 5 to 19% (*4*), where predominant carbohydrates reported are glucose, fructose, and sucrose (*1*). This low caloric fruit (55 cal per 100 g) is also a rich source of vitamins A, E, B9 (folate), B3 (niacin), B1 (thiamine), B2 (riboflavin), B12 (cobalamin) and B5 (pantothenic acid) and D (*10*). The content of vitamin C (ascorbic acid) is significantly higher (16.84 mg) than other tropical fruits like mango (*Mangifera indica* L.; 8.34 mg), carambola (*Averrhoa carambola* L.; 4.67 mg), muskmelon (*Cucumis melo* L.; 2.75 mg), and watermelon (*Citrullus lanatus* L.; 2.38 mg) (*11*). This fact makes papaya highly recommended for persons with iron deficiency anemia. Regular consumption of papaya fruit is advised for preventing vitamin A deficiency, a cause of childhood blindness in tropical and subtropical developing countries (*12*). A half of papaya fruit in general covers the adult minimum daily requirements of vitamins A and C according to the US Food and Nutrition Board (*2*).

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Figure 2. Papaya uses.

The presence of essential amino acids such as tryptophan (4-5 mg), methionine (1 mg) and lysine (15-16 mg) have been reported in papaya. In addition, the fruit provides important minerals (per 100 g) such as calcium (24 mg), magnesium (10 mg), sodium (3 mg), potassium (257 mg), iron (0.1 mg) and boron (0.1-0.2 mg) (*12*). Total phenolic compounds are also reported in papaya fruit (67.8 mg gallic acid equivalent for each 100 g FW) (*11*) that are associated with antioxidative properties. Papain present in papaya fruit helps to improve digestion. Fruit and other organs of papaya have also reported to contain bioactive compounds like flavonoids, cyanogenic glucosides, and glucosinolates with antiinflammation, antiplatelet, antithrombotic, among others mentioned above (*2*). The regular intake of papaya fruit prevents disease like neurodegenerative and cardiovascular illness and may promote the health and well-being of consumers.

Ripening in Papaya Fruit

One important limiting of marketing and export of papaya fruit is the postharvest handling that creates losses up 75%, overripening being one of the most important causes (*4*). Ripening is a complex process that comprises several physical, chemical and biochemical changes in the fruit, including chlorophyll degradation and development of carotene pigments, sweeter taste due to increase in soluble sugars, production of volatile compounds and cell wall disassembly by hydrolase action (*13*).

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These changes are desirable since fruit become edible and palatable and taste and volatile compounds are produced; however, ripening takes in a very short time and once initiated, this irreversible process could induce overripening, susceptibility to pathogens and impair the fruit quality with substantial economical losses (*6*).

Ethylene and Cell Wall Hydrolase Action on Fruit Ripening

Papaya is a climacteric fruit, characterized by a peak in respiration with a coincident burst of ethylene when ripening begins (Figure 3). Ethylene remains to have the most well-defined role in the typical phenotype changes implicated in this process (*14*, *15*).

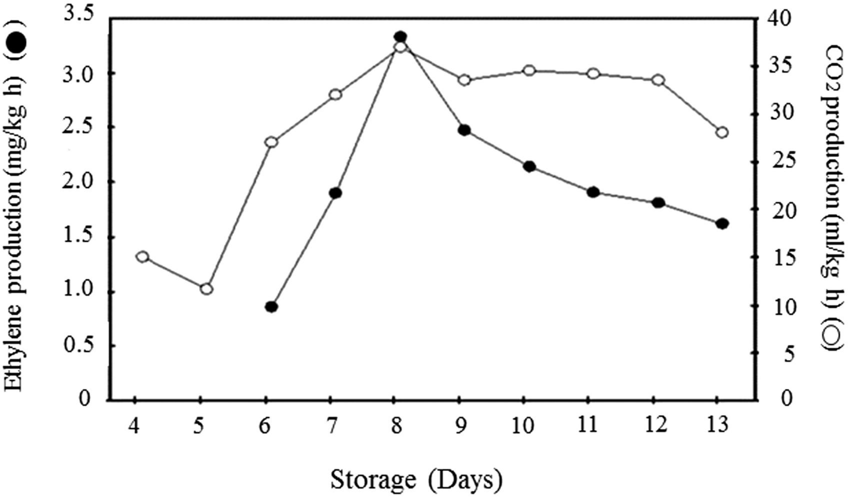

Figure 3. Evolution of respiration (CO_2) and ethylene production in "Maradol" papaya fruits. (Adapted with permission from reference (5). Copyright 2009.)

Genome of papaya recently sequenced, shows the presence of gene related to ethylene synthesis, namely four for S-adenosyl methionine synthase (SAM synthase); eight for 1-aminocyclopropane carboxylic acid (ACC) synthase (ACS); eight for ACC oxidase (ACO) and 42 ethylene-responsive binding factors (*9*). Fruit softening is one of the most prominent ripening changes in climacteric fruits, due to depolymerization and solubilization of cell wall cellulose, hemicelluloses, pectin, and other storage carbohydrates by concerted action of hydrolases (*6*), such as polygalacturonase, pectinmethylesterase, β-galactosidase, endo-1,4-β-D-xylanhydrolase. In addition, expansins with no apparent hydrolytic enzymatic activity, might contribute to cell wall degradation by disrupting hydrogen bonds between cellulose and xyloglucans and increasing the accessibility to hydrolytic proteins (Figure 4) (*16*).

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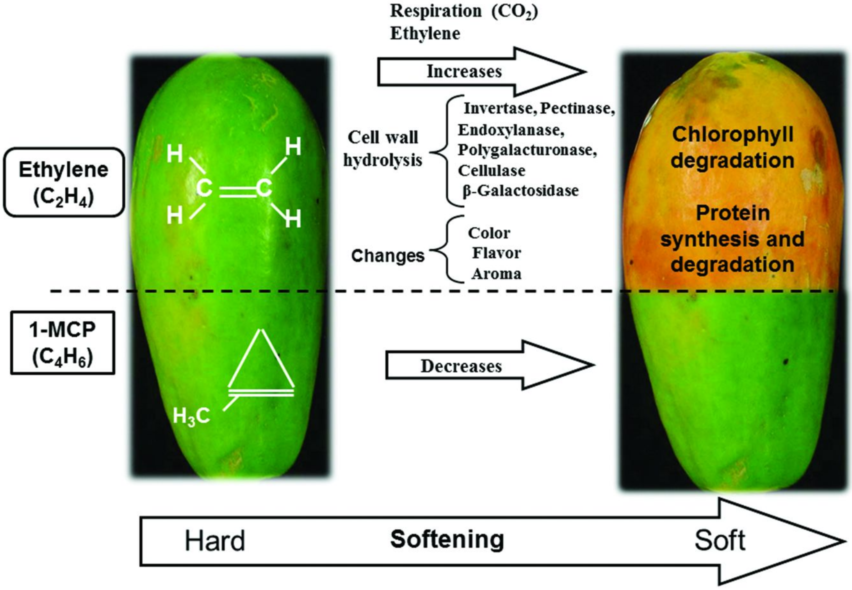
Respiration (CO2)
Ethylene
Increases
Ethylene (C2H4)
H
H
C
C
H
H
Cell wall hydrolysis
Invertase, Pectinase,
Endoxylanase,
Polygalacturonase,
Cellulase
β-Galactosidase
Changes
Color
Flavor
Aroma
Chlorophyll degradation
Protein synthesis and degradation
1-MCP (C4H6)
H3C
Decreases
Hard
Softening
Soft

Figure 4. Ethylene and 1-MCP action on papaya fruit ripening, and biochemical and physiological changes.

The 1-methylcyclopropene (1-MCP) is currently used to reduce ethylene action, as a safe postharvest non-toxic agent for human, animals and environment; it competes with ethylene receptors in climacteric fruits and its application has notable responses including altered ethylene production and respiration, delayed or suppressed softening and altered or delayed volatile emissions (*17*), and repression of enzymes related to fruit respiration and ethylene production (Figure 4).

Recent Ripening Research in Papaya

Ripening research in *C. papaya* L. is relatively scarce and different cultivars show a wide variation in fruit softening (*18*). During normal ripening with "Maradol" papaya fruit the rate of CO_2 production ranged from 4 to 10 µg/kg s, with a peak at 4 d, with ethylene peak at 7 d; those parameters are affected when 1-MCP or exogenous ethylene are applied (*19*).

Application of 1-MCP delayed fruit softening and all hydrolases activities were affected. Xylanase activity was low or undetectable; authors suggest that this might be determinant in papaya softening, since 1-MCP-treated fruit failed to soften completely and mesocarp presented a rubbery texture (*18*).

Endoxylanase activity was measured in "Maradol" papaya at green-mature, half-ripe and ripe fruit (0, 50 and 100% skin yellow, respectively, Figure 5), using Remazol brilliant blue-xylan (RBB-Xylan) as substrate. Its activity was correlated with fruit ripeness. Thus, at 4 h of reaction, in green-mature fruit a low relative

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activity of 4.8% was detected that significantly increased at the half-ripe fruit. The highest enzymatic activity was found in ripe papaya (88.7%), being 1.77-fold higher than that of the half-ripe stage (Figure 5).

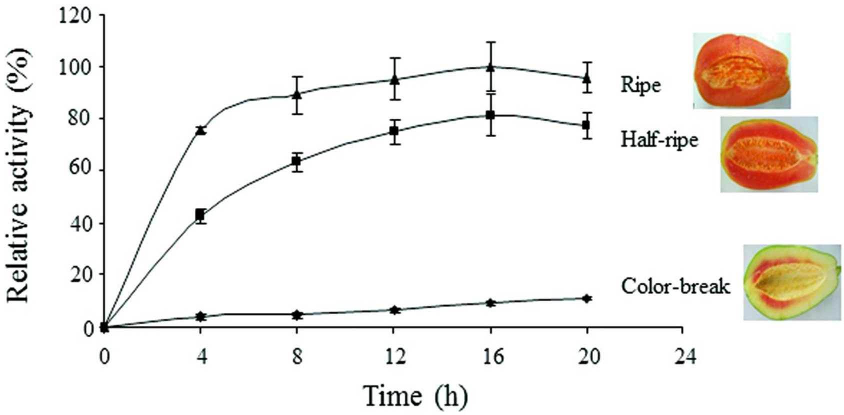

Figure 5. Endoxylanase activity of protein extracts of "Maradol" papaya in three stages of ripening at different reaction time (h), 37 °C and pH 5.4. 100% relative activity is referred as maximum activity (0.0208 μg RBB/ μg protein h) found in ripe papaya at 16 h (mean ± S.D., n = 3).

Electrophoretic analysis of total proteins and immunodetection by western blot using polyclonal antibody from "Sunset" endoxylanase, showed an inactive pre-endoxylanase at 63.9 kDa in the color-break fruit and two probable mature endoxylanases found in ripe papaya: at 32.5 kDa and at 27.4 kDa, a novel enzyme not previously described in this fruit (Figure 6).

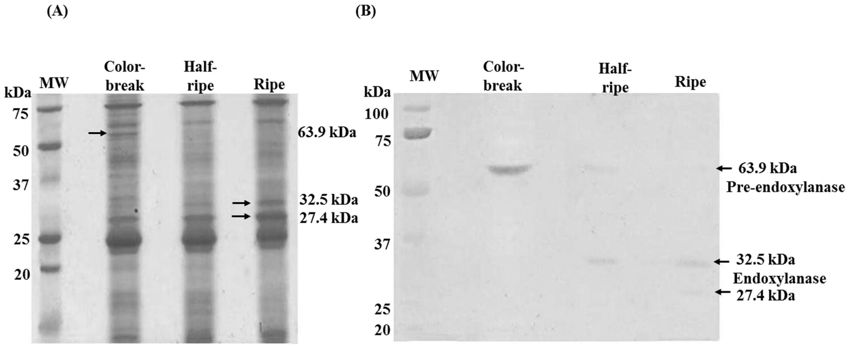
(A)
kDa
MW
Color-break
Half-ripe
Ripe
75
50
37
25
20
63.9 kDa
32.5 kDa
27.4 kDa
(B)
kDa
MW
Color-break
Half-ripe
Ripe
100
75
50
37
25
20
63.9 kDa
Pre-endoxylanase
32.5 kDa
Endoxylanase
27.4 kDa

Figure 6. Identification on tricine SDS-PAGE (A) and by western blot (B) of inactive pre-endoxylanase and mature endoxylanase at differentripening stages of "Maradol" papaya fruits.

In half-ripe fruit two bands at 63.9 and 32.5 kDa were identified, corresponding to the expected pre-endoxylanase and active endoxylanase molecular weights, respectively. Therefore, the enzymatic activity together with immunoblotting results, suggest that the endoxylanase is processed from the 63.9-kDa protein precursor to an active form at 32.5 kDa by a protease cleavage.

The two mature endoxylanases were semipurified by 85% ammonium sulfate precipitation and subsequently two-step chromatographic procedure (carboxymethyl-Sepharose ion exchange and Sephacryl S-200 gel filtration) (*20*). The semipurified endoxylanase had an optimum pH of activity of 5.5 (Figure 7A). The enzyme showed an optimal temperature from 45-50 °C (Figure 7B).

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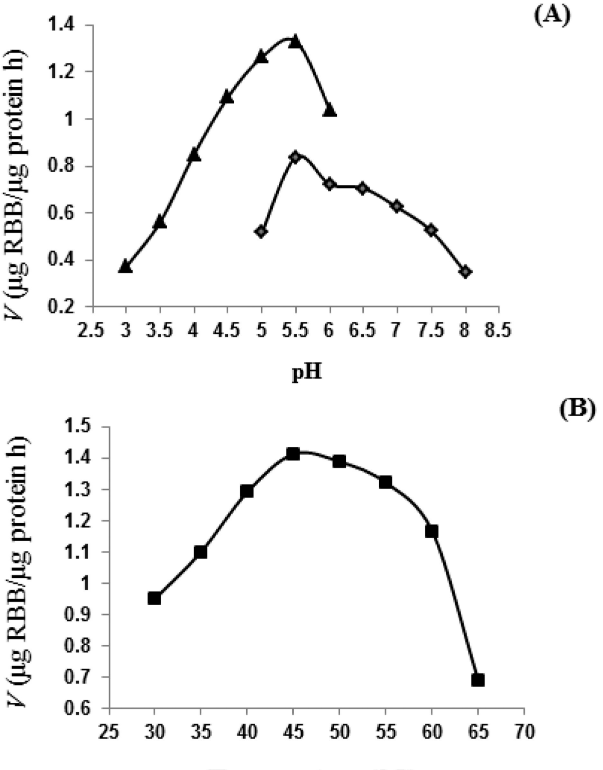

Figure 7. Semipurified endoxylanase activity from ripe "Maradol" measured at different pH values in presence of either 50 mM sodium acetate (filled triangles) or 50 mM dipotassium phosphate (filled squares) (A) and temperature (B) (mean of n = 3). (Adapted with permission from reference (20). Copyright 2012.)

The "Maradol" papaya endoxylanase shows similar optimal enzymatic activity conditions of pH and temperature to that from "Sunset" variety reported by Chen and Paull (*21*); however, "Sunset" papaya activity is less influenced by the pH than by temperature conditions.

A Proteomic Approach

The comparative proteomics approach is a valuable tool to gain insights on the papaya fruit ripening and the biochemical modifications caused by the inhibition of ethylene action (*22*). Huerta-Ocampo et al. (*23*) conducted a research work in order to study, the protein changes in "Maradol" papaya fruits during natural ripening in comparison with fruits treated with 1-MCP.

A group of differentially expressed proteins were associated to simple and complex sugar metabolism. Two cell wall invertases (spots 1 and 2) with different isoelectric points (p*I*) were significantly increased after 6 d of natural ripening, but decreased in abundance in presence of 1-MCP. Two pectinesterases (PEs) with different p*I* were also identified, one (spot 14) significantly increased, while other (spot 15) decreased after 18 d of natural ripening. It is known that multiple isoforms of PEs exist in fruit tissues, but it is still unclear why plant makes more than one isoform of this enzyme (*24*), but also this could be associated to isoforms sensitive and insensitive to ethylene. One endoxylanase of 34 kDa and neutral p*I* of 7.08 (spot 13) was identified. This protein significantly increased only under natural ripening in papaya fruits.

The increase in disease resistance during ripening is another postharvest concern. Osmotin is an abundant cationic multifunctional protein (*25*), and belongs to the PR-5 family of plant defense proteins (*26*). One osmotin in papaya fruits was absent in control (day 0) and increased in abundance with the progression of natural ripening, however in 1-MCP treated fruits it was not observed until 18 days of postharvest (Table I).

Reactive oxygen species play key roles in the activation of disease resistance mechanisms in plants (*27*). Two different SOD^{Cu-Zn} were identified among the differentially expressed proteins; one of them (spot 6), was significantly increased (Table I) after 6 d and 18 d of natural ripening; but no significant changes were observed in fruits treated with 1-MCP. Meanwhile, the second (spot 7) was significantly increased under both treatments. up-accumualted after 6 d of postharvest (*23*). Total SOD activity has been related to a longer commercial life and higher resistance to stress (*28*, *29*).

Glutathione S-transferases (GSTs) can function as a H_2O_2-scavenging mechanism and three different GSTs were identified among the differentially accumulated protein spots (spots 4, 9, and 22). Abundance of spot 4 was not significantly increased after 6 d under natural ripening, while spots 9 and 22 significanlty increased in all treatments. In all cases, the highest amount was observed after 18 days in 1-MCP treated fruits. It has been observed that the treatment with 1-MCP in pears, apples, and peaches promoted SOD and POX activities (*30*–*32*).

Spot 16 (Table I) was identified as an aldo/keto reductase (AKR) and increased mainly after natural ripening. It has been sugested that AKR is involved in the stress/hormone response of plants (*33*). Dienelactone hydrolases play a crucial role in the bacterial degradation of chloraromatic compounds and could be activated by H_2O_2 or HCN compounds, they have also been claimed as a new target for thioredoxin (*34*, *35*). Three isoforms of dienelactone hydrolase (spots 21, 22, and 23) showing differential accumulation patterns were identified.

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Table I. Proteins associated with sugar metabolism, and defense and stress responses, their identities and relative changes in "Maradol" papaya pulp during natural ripening and in response to 1-MCP treatment. (Adapted with permission from reference (*23*). Copyright 2012)

Spot[a]	*Protein name*	*Accession number*[b]	M_r/pI[c]	*Differences in protein abundance*[d]			
				A	*B*	*C*	*D*
Sugars and cell wall metabolism							
1	Cell wall invertase	gi|16225878	75.0/6.58	↑	↓	Ns	↓
2	Cell wall invertase	gi|16225878	75.0/6.70	↑	↓	Ns	↓
14	Pectinesterase	EX249434	22.0/4.84	Ns	↓	↑	Ns
15	Pectinesterase	EX249434	21.7/5.04	Ns	↓	↓	↑
13*	Endoxylanase	gi|23429644	34.0/7.08	↑	Ns	↑	Ns
Defense and stress responses							
26	Osmotin	EX255680	23.7/7.43	↑	¶	↑	↑
6	Superoxide dismutase	gi|12230571	16.2/6.21	↑	Ns	↑	Ns
7	Superoxide dismutase	gi|58615981	14.8/5.80	↑	↑	↑	↑
4	Glutathione S-transferase	EX301201	25.0/6.50	Ns	↑	↑	↑
9	Glutathione S-transferase	EX254349	26.7/5.49	↑	↑	↑	↑
22*	Glutathione S-transferase	EX254349	26.7/5.49	↑	↑	↑	↑
16	Aldo/keto reductase	EX257277	25.5/7.52	↑	Ns	↑	↑
21	Dienelactone hydrolase	EX276208	28.3/5.49	Ns	↑	Ns	↑
22*	Dienelactone hydrolase	EX276208	26.7/5.63	↑	↑	↑	↑
23	Dienelactone hydrolase	EX273694	26.3/5.79	Ns	Ns	Ns	↑
17	Translationally controlled tumor protein	gi|255626449	12.0/9.30	Ns	↓	↑	Ns

[a] Spot numbers [b] Accession numbers according to Viridiplantae nrNCBI database and Plants EST Database of NCBI. [c] Experimental mass (kDa) and pI of identified proteins. [d] Differences in protein abundance with respect to control at day 0. Columns A and C samples at 6 and 18 days after natural ripening; Columns B and D, treatment with 1-MCP at 6 and 18 days. Ns = no significant; ↑ = significantly increased spots; ↓ = significantly decreased spots ¶ = Spot not present.

The translationally controlled tumor protein (TCTP) is highly regulated in response to various cellular stimuli and stresses (*36*). During "Maradol" papaya fruit ripening, TCTP was increased significantly after 18 d of postharvest in control fruits. Changes in proteins in postharvest citrus fruit are apparently related to the activation of programmed cell death (*37*).

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Conclusions

Biochemical characteristics of endoxylanase presented in “Maradol” fruit are crucial to our understanding of fruit softening mechanisms. However, the patterns of expression during its post-translation processing, and its involvement in cell wall hydrolysis and fruit softening during the complex process of maturation needs further research. As a result of 1-MCP treatment, the inhibition of the enzymes responsible of the cell wall degradation was observed. On the other hand, the role of different forms of the same protein showing differential abundance profiles during ripening as in the case of PEs, SODs, and dienelactone hydrolase family proteins deserve further research.

Papaya fruit softening is a complex process where hydrolytic and non-hydrolytic proteins are involved and expressed differentially during ripening. Recent research points to biochemical characterization of cell wall hydrolases, in particular the endoxylanase, and the participation of proteins traditionally related with defense and redox cell metabolism as crucial to understanding this phenomenon. Biotechnological strategies aimed to extend the shelf life of papaya without loss of either nutritional and sensorial properties, might be essential to increase the availability of nutraceutical compounds contained in papaya fruit in tropical and non-tropical countries.

Acknowledgments

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Chapter 6

Inhibition of α-Glucosidase and α-Amylase by *Vaccinium floribundum* and *Aristotelia chilensis* Proanthocyanidins

Maria Schreckinger, Mary Ann Lila, Gad Yousef, and Elvira de Mejia*

Division of Nutritional Scienes, University of Illinois,
228 ERML, Urbana, IL 61801
***E-mail: edemejia@illinois.edu**

Extracts from *Vaccinum floribundum* and *Aristotelia chilensis*, two promising South American berry species, were characterized in terms of their phytochemical composition and ability to inhibit α-glucosidase and α-amylase activities *in vitro*. *A. chilensis* contained 45.7 mg/g DW total anthocyanins (cyanidin-3-glucososide (C3G) equivalents). Seven anthocyanins were identified in this berry, of which delphinidin-3-glucoside was the main anthocyanin. *A. chilensis* contained proanthocyanidin dimers (56%) and trimers (14%). *V. floribundum* was comprised of trimers (68%) and in less proportion pentamers (16%) and hexamers (8%). *V. floribundum* proanthocyanidins inhibited both α-glucosidase (IC_{50}=35 μg/mL) and α-amylase (IC_{50}=25 μg/mL), which warrants further research into potential dietary use of this berry in therapies to combat diabetes symptoms.

Introduction

A multiplicity of health-promoting bioactivities have been linked to berry fruits, encouraging wider consumption worldwide (*1*). While the medicinal benefits of berry fruits have been recognized for centuries as part of the traditional ecological knowledge of tribal nations in North America and throughout the world (*2*, *3*), it is only recently that the bioactive principles accumulated within berries, and their mechanisms of action after ingestion and metabolism by animals

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(including humans), have been elucidated in multiple *in vitro*, *in vivo*, and now clinical trials (*4–7*).

The most prevalent bioactive constituents in these fruits are the polyphenolics; principally the flavonoid class of anthocyanins (the red and blue pigments) and proanthocyanidins. However, increasingly, the apparent incidence of potentiating interactions between fruit phytochemicals (anthocyanins, proanthocyanidins, phenolic acids, etc.), to intensify a bioactive effect, have been demonstrated (*8–10*), which suggests that consumption of whole fruits, rather than individual extracted compounds in the form of supplements, is more protective for health maintenance.

A particularly strong source of leads for medicinally-active principles from plants has been wild-crafted plant populations, especially those plants indigenous to particular areas of the world, and already recognized for their health-promoting properties by local communities (*2*, *11*). For berries, the principal mechanism of action has been for a long time assumed to be antioxidant capacity, however, recent research indicates that other complementary mechanisms of action, including anti-inflammatory activity, signaling, and enzyme regulation, are contributed by berry fruit constituents (*12–14*).

South American berries, a new exotic introduction to the global marketplace, have proven to be particularly rich in bioactive flavonoids, and offer an intriguing and novel resource for proactive dietary maintenance for human health (*15–17*). This report demonstrates the *in vitro* inhibition of alpha glucosidase and alpha amylase enzymes by phenolic extracts (particularly proanthocyanidins) of two promising South American berry fruits, *Vaccinium floribundum* (mortino) and *Aristotelia chilensis* (maqui berry), indicative of potential utility as anti-diabetic agents.

Materials and Methods

Berry Collection and Storage

Ripe *Aristotelia chilensis* berries were collected in January 2009 from the Entrelagos region in Chile (S 40º 40’ 48.5"/ W 72º 33’ 43.3"). Ripe *Vaccinium floribundum* berries were collected during late November 2008 in the grasslands of Simiatug, Ecuador. The berries were cleaned by removing leaves, stems and damaged berries. The whole berries were freeze-dried, sealed in plastic bags, and shipped to our laboratory. The berries were stored at -80 ºC until usage.

Preparation of Phenolic-Rich Extracts and Fractions

The process used to prepare the phenolic extracts and enriched fractions (Figure 1) was based on procedures developed by Grace et al. (*18*). For the preparation of the phenolic-rich extract, the freeze dried berries were blended with 80% ethanol acidified with 0.3% trifluoroacetic acid (TFA). The collected hydro-alcoholic extract was evaporated using a rotary evaporator at a temperature not exceeding 40 °C. The aqueous concentrate was partitioned with ethyl acetate (4 × 500 mL) to remove lipophilic material. The aqueous layer was retained and loaded onto an Amberlite XAD-7 column (30 × 10 cm). The resin was washed thoroughly with acidified water (0.3% TFA, ~3 L) to remove free sugars, pectins, and phenolic acids. The polyphenolic mixture was then eluted with acidified ethanol (0.3% TFA), evaporated, and freeze-dried to yield post-amberlite extract (PAE). For the preparation of the anthocyanin and proanthocyanidin-enriched fractions, 2 g of the PAE were placed on a Sephadex LH-20 column (30 × 3 cm). Anthocyanins were obtained from an isocratic elution of 20% aqueous ethanol acidified with 0.3% TFA. The column was then washed with 70% aqueous acetone to elute polymeric proanthocyanidins. All fractions were concentrated and freeze-dried to yield the anthocyanin-enriched (ANC) or proanthocyanidin-enriched (PAC) fraction.

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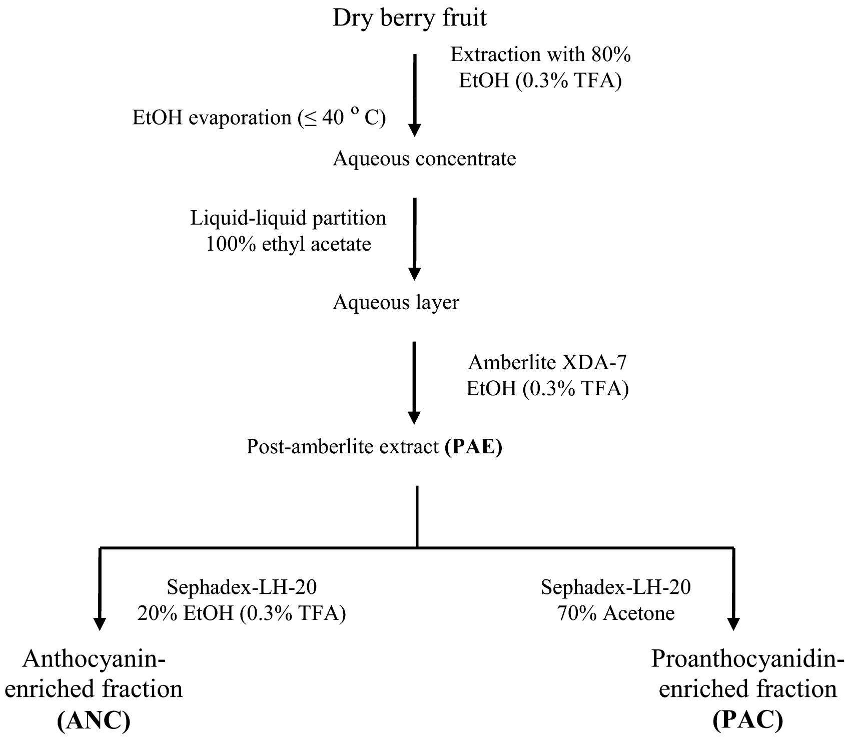
Dry berry fruit
Extraction with 80% EtOH (0.3% TFA)
EtOH evaporation (≤ 40 ° C)
Aqueous concentrate
Liquid-liquid partition 100% ethyl acetate
Aqueous layer
Amberlite XDA-7 EtOH (0.3% TFA)
Post-amberlite extract (PAE)
Sephadex-LH-20 20% EtOH (0.3% TFA)
Sephadex-LH-20 70% Acetone
Anthocyanin-enriched fraction (ANC)
Proanthocyanidin-enriched fraction (PAC)

Figure 1. Extraction of anthocyanin and proanthocyanidin enriched fractions. Abbreviations: PAE, post-amberlite extract; ANC, anthocyanin-enriched-fraction; PAC, proanthocyanidin-enriched-fraction. (Adapted with permission from reference (18). Copyright 2009 Elsevier.)

Anthocyanin and Proanthocyanidin Analysis

Anthocyanin and proanthocyanin separation was conducted on an 1100 HPLC (Agilent Technologies, Santa Clara, CA) using a reverse phase Supelcosil-LC 18 column (250 mm × 4.6 mm × 5 μm) (Supelco, Bellefonte, PA). Anthocyanins

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and proanthocyanins were detected at 520 nm and 280 nm using diode array detector (DAD), respectively. Chemstation software (Agilent Technologies Inc, Santa Clara, CA) was used for both protocol control and data processing. The HPLC-ESI-MS analyses were made with an LCQ Deca XP mass spectrometer (Thermo Finnigan Corp., San Jose, CA). The HPLC separations were carried out on a C-18 reversed-phase column (150 mm, 2.1 mm i.d., particle size 5 µm, 90Å) (VYDAC, Western Analytical, Murrieta, CA, USA). Acquisition of LC-PDA-MS data was performed and processed using XCalibur Qual Browser v1.4 software (Thermo Electron Corp., Waltham, MA). The total anthocyanin content of the two berries (*A. chilensis* and *V. floribundum*) was calculated as cyanidin-3-glucoside equivalent (C3G). Three concentrations of the standard at 0.25 mg, 0.5 mg, and 1.0 mg/mL were used to quantify the anthocyanins using peak areas measured by HPLC at 520 nm. The identification of anthocyanins was based on comparison with published data (*18–21*), MS spectral data and comparison to reference standards. In the same way, the total proanthocyanidin content of the two berries was calculated as epicatechin equivalents from the peak area measured at 280 nm, with the exclusion of 520 nm wavelength peaks which were anthocyanins.

α-Glucosidase and α-Amylase Inhibition

The enzymatic inhibition assays were adapted from Apostolidis et al. (*22*). For the α-glucosidase assay, in a 96-well plate 50 µL of sample (50 µg/mL of phenolic extract), standard or blank was added to 100 µL of a1U/mL α-glucosidase solution (in 0.1 M sodium phosphate buffer pH, 6.9) and incubated for 10 min. A 50 µL aliquot of a 5 mM p-nitrophenyl-R-D-glucopyranoside solution (in 0.1M sodium phosphate buffer pH, 6.9) was added to each well and incubated at 25 °C for 5 min before the absorbance was read at 405 nm. For the α-amylase assay, 500 µL of either sample (50 µg/mL of phenolic extract), standard or blank was added to 500 µL of 13 U/mL α-amylase solution (in 0.02M sodium phosphate buffer, pH 6.9) and incubated in test tubes at 25 °C for 10 min before the addition of 500 µL of 1% soluble starch solution (dissolved in sodium phosphate buffer and boiled for 10-15 min). The mixture was incubated for another 10 min before 1 mL of dinitrosalicylic acid color reagent was added. The tubes were placed in 100 °C water for 5 min and then the mixture was diluted with 10 mL of distilled water. Absorbance was read at 520 nm.

Statistical Analysis

Data were expressed as means of at least two independent replicates. Results were compared by one-way analysis of variance (ANOVA) using the Proc GLM function of SAS version 9.2 (SAS Inst. Inc., Cary, NC). Group means were considered to be significantly different at $p < 0.05$. Mean separation was achieved through least significant difference (LSD) procedure in SAS.

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Results and Discussion

Phenolic Composition

Table 1 shows the total anthocyanins and total proanthocyanidins of *A. chilensis* and *V. floribundum* berries and their phenolic extracts. HPLC analysis indicated that the total anthocyanin and proanthocyanidin content of *A. chilensis* was 45.7 mg/g DW (C3G equivalent) and 4.0 mg/g DW (epicatechin equivalents), respectively. The berries of *V. floribundum* contained 10.6 mg/g DW (C3G equivalents) of total anthocyanins and 5.2 mg/g DW (epicatechin equivalents) of total proanthocyanidins.

Table 1. Total anthocyanins and total proanthocyanidins of *Aristotelia chilensis* and *Vaccinium floribundum* berries and phenolic extracts

	Anthocyanins (%)[1]		***Proanthocyanidins (%)***[2]	
	A. chilensis	*V. floribundum*	*A. chilensis*	*V. floribundum*
DB	4.7 ± 0.1[3]	1.1 ± 0.1[3]	0.4 ± 0.3[3]	0.5 ± 0.1[3]
PAE	58.4 ± 0.7	11.1 ± 0.5	5.1 ± 0.4	5.3 ± 0.5
ANC	79.8 ± 1.7	15.7 ± 0.2	≥ 0.1	≥ 0.1
PAC	≥ 0.1	≥ 0.1	49.3 ± 3.2	54.3 ± 2.4

[1] % expressed as cyanidin-3-glucoside equivalents; [2] % expressed as epicatechin equivalents. [3] Data from phenolic-rich extracts was converted to dried fruit weight basis. Abbreviations: DB, dry berry; PAE, post-amberlite extract; ANC, anthocyanin-enriched-fraction; PAC, proanthocyanidin-enriched-fraction.

Anthocyanins

The berry of *A. chilensis* contained seven main anthocyanin structures: delphinidin-3-sambubioside-5-glucoside (m/z: 759), delphinidin-3,5-diglucoside (m/z: 627), cyanidin-3-sambubioside-5-glucoside (m/z: 743), delphinidin-3-sambubioside (m/z: 597), delphinidin-3-glucoside (m/z: 465), cyanidin-3-sambubioside (m/z: 581), and cyanidin-3-glucoside (m/z: 449). This was in agreement with previous reports (*18–20*). An HPLC chromatogram illustrating these structures in the PAE is shown in Figure 2A and the peak assignment and the concentration of individual anthocyanins in the PAE and in the ANC for *A. chilensis* is presented in Table 2. Delphinidin 3-glucoside (peak 5) was the main anthocyanin component present in this berry. In the berries of *V. floribundum* five anthocyanins were identified: delphinidin-3-galactoside (m/z 465), cyanidin-3-galactoside (m/z 449), delphinidin-3-arabinose (m/z 435), cyanidin-3-glucoside (m/z 449), and cyanidin-3-arabinose (m/z 419) (Figure 2B). This was in agreement with previous studies (*21*). Delphinidin-3-arabinose (peak 2) and cyanidin-3-arabinose (peak 5) were found to be the main anthocyanins in this berry. The peak assignment and the individual concentrations of anthocyanins present in the PAE and ANC of *V. floribundum* are presented in Table 2.

Table 2. Identification and percent of anthocyanins in the post-amberlite extract (PAE) and anthocyanin-enriched-fraction (ANC) of *Aristotelia chilensis* and *Vaccinium floribundum*

Peak	*Anthocyanin*	*PAE (%)*	*ANC (%)*
Aristotelia chilensis			
1	Delphinidin-3-sambubioside-5-glucoside	8.4	11.9
2	Delphinidin-3,5-diglucoside	7.5	11.0
3	Cyanidin-3-sambubioside-5-glucoside	6.1	8.7
4	Delphinidin-3-sambubioside	9.3	12.9
5	Delphinidin-3-glucoside	17.5	22.3
6	Cyanidin-3-sambubioside	0.3	0.4
7	Cyanidin-3-glucoside	9.3	12.5
Vaccinium floribundum			
1	Delphinidin-3-galactoside	1.0	1.4
2	Cyanidin-3-galactoside	4.8	7.0
3	Delphinidin-3-arabinose	1.0	1.4
4	Cyanidin-3-glucoside	0.4	0.4
5	Cyanidin-3-arabinose	3.9	5.5

Percentages were calculated as cyanidin-3-glucoside equivalents. Abbreviations: post-amberlite extract (PAE), anthocyanin-enriched-fraction (ANC).

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Proanthocyanidins

HPLC-MS analysis revealed a series of proanthocyanidins ranging from dimers to hexamers. *A. chilensis* contained mainly dimers (56%) and trimers (14%). Small MS peaks of proanthocyanidin tetramers, pentamers, and hexamers were also detected. *V. floribundum* contained trimers (68%) and in less proportion pentamers (16%) and hexamers (8%). Figure 3 illustrates the UV chromatogram, at 280 nm absorption and the ESI/MS spectra of the PAC fraction of *V. floribundum*. The ESI/MS spectra illustrates a series of proanthocyanidins ranging from dimers to hexamers. In the UV chromatogram a large hump characteristic of proanthocyanidins can be seen.

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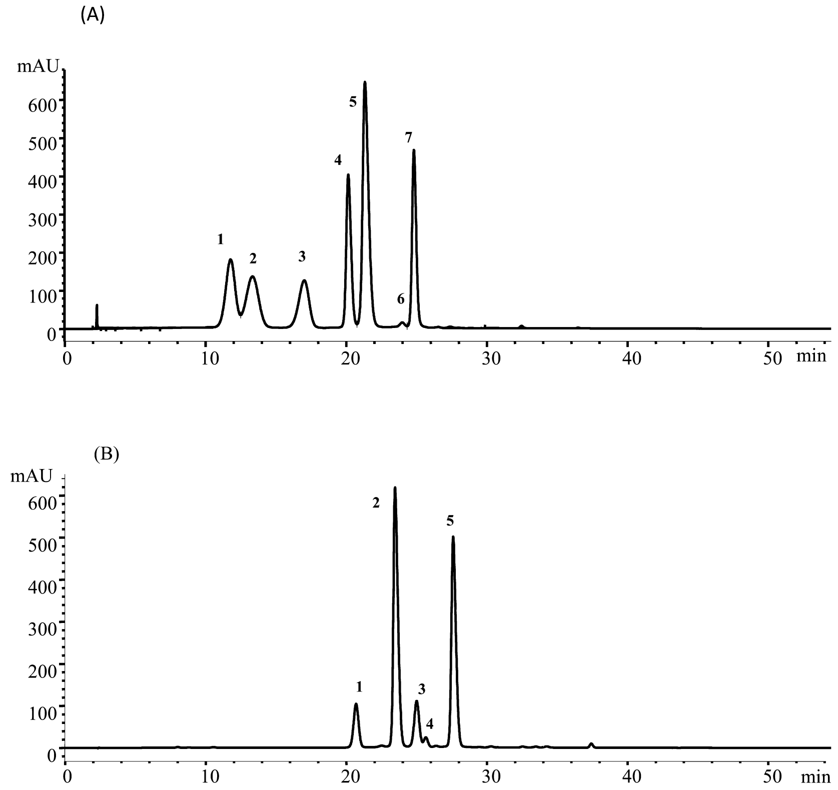

Figure 2. Chromatographic profiles of post-amberlite extract (PAE) of Aristotelia chilensis (A) and Vaccinium floribundum (B). Identities of these compounds are presented in Table 2.

Inhibitory Capacity of Phenolic Extracts on α-Glucosidase and α-Amylase

Tables 3 and 4 show the percent inhibition and IC_{50} values of α-glucosidase and α-amylase respectively, after treatment with 50 µg/mL of phenolic extracts. Five of the six phenolic extracts inhibited the activity of α-glucosidase and this inhibition ranged from 4.4 to 99.3 % (Table 3). The PAC fraction of *V. floribundum* and *A. chilensis* were the most potent inhibitors (99.3-98.8% respectively), both showing an IC_{50} < 25 µg/mL. For α-amylase, three of the six phenolic extracts inhibited the activity of this enzyme, and this inhibition ranged from 11.7 to 53.6 % (Table 4). The PAC fraction of *V. floribundum* was the most potent inhibitor with an IC_{50} of 31.4 (µg/mL). These results show that PAC fractions of both berries, in particular the PAC fraction of *V. floribundum*, are potent inhibitors *in vitro* of α-glucosidase and α-amylase. The results of this study show that the phenolic extracts of *V. floribundum* and *A. chilensis*, in particular the proanthocyanidin rich fractions, have the potential to reduce glucose absorption by inhibiting α-glucosidase and α-amylase involved in starch degradation. These berries could potentially be used as a therapeutic approach for the management of type 2 diabetes in a dietary regime.

Table 3. Inhibition (%) and IC_{50} values of α-glucosidase exposed to 50 µg/mL of phenolic extracts

	Phenolic Extract	*Inhibition (%)*	*IC_{50} (µg/mL)*
A. chilensis	**Post- amberlite extract (PAE)**	73.2 ± 2.5	35.9
	Anthocyanin-enriched (ANC)	4.4 ± 0.9	> 100
	Proanthocyanidin-enriched (PAC)	98.8 ± 0.3	< 25
V. floribundum	**Post- amberlite extract (PAE)**	56.4 ± 5.5	52.6
	Anthocyanin-enriched (ANC)	-1.6 ± 1.7	> 100
	Proanthocyanidin-enriched (PAC)	99.3 ± 0.03	< 25

The data represents the mean ± SD from at least two independent studies and a least a triplicate analysis.

Table 4. Inhibition (%) and IC_{50} values of α-amylase exposed to 50 µg/mL of phenolic extracts

	Phenolic Extract	*Inhibition (%)*	*IC_{50} (µg/mL)*
A. chilensis	**Post- amberlite extract (PAE)**	11.7 ± 3.1	> 100
	Anthocyanin-enriched (ANC)	-11.8 ± 1.2	> 100
	Proanthocyanidin-enriched (PAC)	46.6 ± 3.3	> 100
V. floribundum	**Post- amberlite extract (PAE)**	-33.8 ± 0.5	> 100
	Anthocyanin-enriched (ANC)	-71.4 ± 1.6	> 100
	Proanthocyanidin-enriched (PAC)	53.6± 6.6	31.4

The data represents the mean ± SD from at least two independent studies and a least a triplicate analysis.

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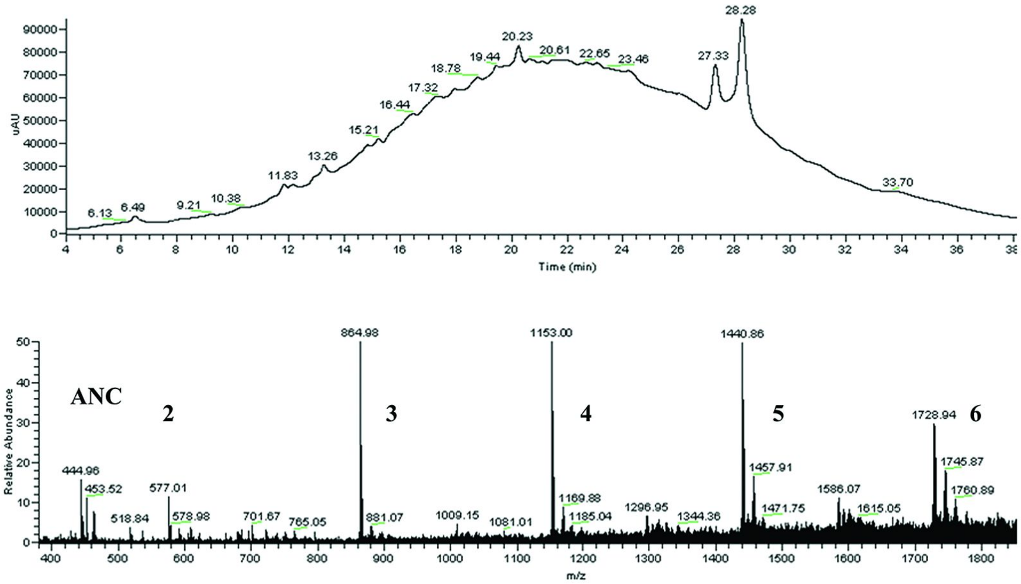

Figure 3. HPLC –ESI/MS profiles of proanthocyanidin-enriched-fraction (PAC) of V. floribundum including UV chromatogram with 280 nm absorption and ESI/MS spectra. The large bold numbers indicate the average degree of polymerization from dimers to hexamers and ANC refers to anthocyanins.

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Figure 4 presents examples of Ecuadorian commercial products such as a dry powder ready to be incorporated into everyday dishes. This figure also shows a traditional preparation based on Mortino *Vaccinium floribundum* called "colada morada". Mortino is also commonly used to produce jams, sauces, and even wines.

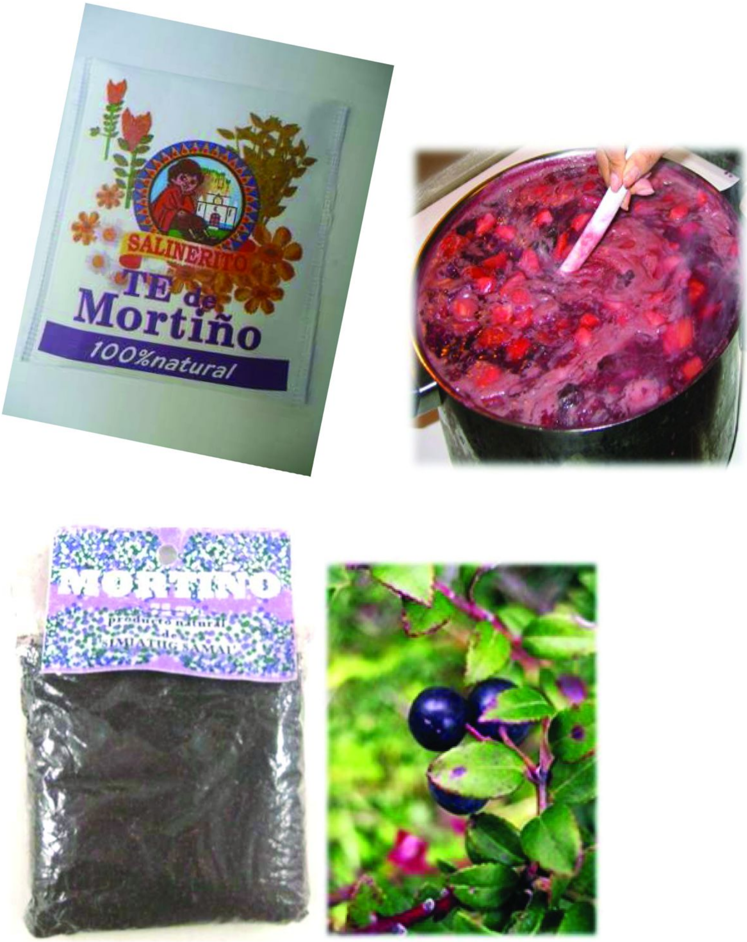

Figure 4. Examples of Ecuadorian commercial products and traditional dish based on Mortino Vaccinium floribundum.

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Conclusions

- Post-amberlite (PAE) and proanthocyanidin-enriched (PAC) extracts from both berry species inhibited α-glucosidase activity.
- Proanthocyanidin-enriched (PAC) extracts of both berry species inhibited α-amylase activity.
- Only the proanthocyanidin-enriched (PAC) extract of *V. floribundum* was a potent inhibitor of both α-glucosidase and α-amylase activity.

Both *A. chilensis* and *V. floribundum* contain phytochemicals that may be relevant to dietary interventions to curtail diabetes symptoms.

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Chapter 7

Huitlacoche – A 21st Century Culinary Delight Originated in the Aztec Times

María Elena Valverde, Talía Hernández-Pérez, and Octavio Paredes-Lopez*

**Centro de Investigación y de Estudios Avanzados-IPN,
Unidad Irapuato, Km 9.6 Libramiento Norte Carretera
Irapuato-León, Irapuato, Guanajuato 36821, México
*E-mail: oparedes@ira.cinvestav.mx**

Common smut or boil smut are the young, fleshy, edible galls produced in the ears of maize (*Zea mays*) when they are infected by the fungus *Ustilago maydis*. *U. maydis* belongs to the Ustilaginales order and has been established as a robust pathogenic model to study fungi and fungi–plant relationships; also, it has been transformed and its genome sequenced. Cuitlacoche or huitlacoche is the Aztecs name given to these galls. In México, it has been traditionally prized and several hundreds of tons of fresh huitlacoche are sold annually; it is also consumed as prepared food or processed in Mexican markets. Currently, it has been appreciated in the US as a gourmet food and can be purchased on the Internet at high prices. Nowadays, it is a culinary delight for international chefs and has been accepted as a food delicacy in many countries and introduced into countless worldwide markets like Japan, China and some of the European Community, as France, Spain and Germany. In addition to its unique flavor, huitlacoche has been identified as a high-quality functional food, and could be included into the daily diet for its attractive characteristics, selected nutrients, valuable compounds, and nutraceutical potential.

In Hispanic Foods: Chemistry and Bioactive Compounds; Tunick, M., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

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Introduction

In México and some other Latin American countries, young smut tumors growing on immature corn ears are consumed as main dish or condiment and are highly appreciated as a food delicacy (Figure 1) (*1*, *2*). Huitlacoche is the native Mexican name given to these young, fleshy, edible galls produced when the ears of maize (*Zea mays*) are infected by *Ustilago maydis*; however, throughout the world these galls are best known as common smut or boil smut (Figure 2). Huitlacoche or cuitlacoche comes from the nahuatl (prehispanic Mexican language) “cuitlacochin” or “cuitlacuchtli” that means “degenerated corn on the cob”, and has been traditionally prized as a delicacy for various international chefs due to its unique flavor, aroma and sensory characteristics. In addition, huitlacoche has interesting nutrimental content and health-promoting compounds, like proteins, amino acids, dietary fibers, unsaturated fatty acids, and specifically β-glucans among other carbohydrates (*2*–*4*).

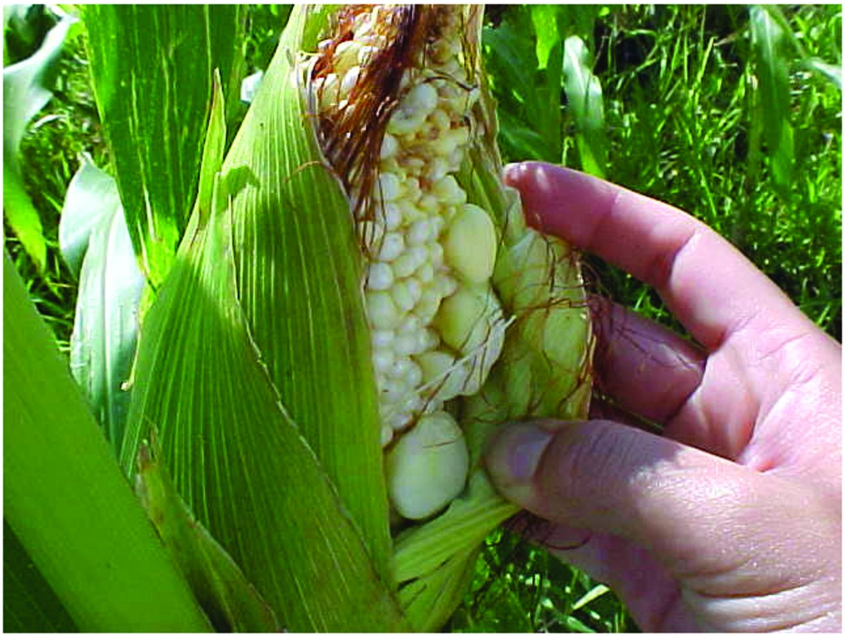

Figure 1. Young smut tumors growing on immature corn ears.

U. maydis belongs to the Ustilaginales order that includes semi-obligate biotrophic plant pathogenic fungi with a very narrow host range because infects only maize and its progenitor plant teosinte (*Zea mays* subsp. *parviglumis*). It is a heterothallic fungus with a tetrapolar mating system and a dimorphic life cycle with a saprophytic and a parasitic phase. In nature, pathogenic and sexual development is connected in *U. maydis* and it entirely depends on its host to complete its life cycle (*5*, *6*).

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On the other hand, *U. maydis* has been found as a robust pathogenic model to study fungi and fungi–plant relationships, especially because of the morphological transitions throughout its life cycle, easy culture, genetic manipulation in the laboratory, mating type, biotrophic host interaction, as well as the genetic properties to elucidate the molecular mechanisms of the interaction between plant and pathogen and the severe disease symptoms it induces in infected maize (*7*, *8*).

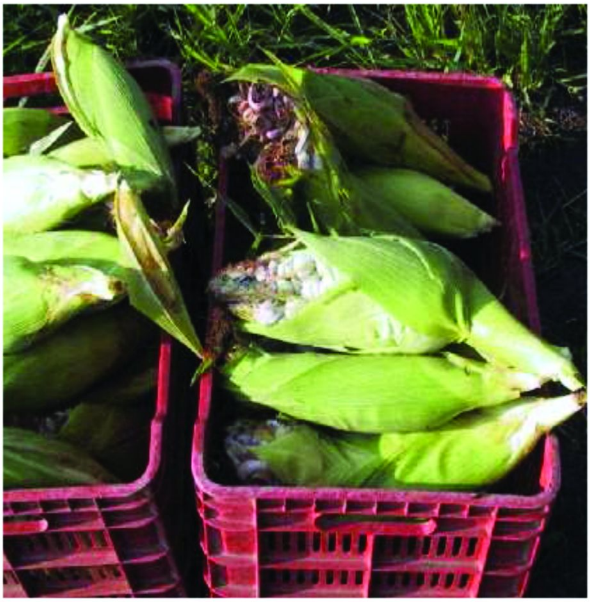

Figure 2. Common smut or huitlacoche.

U. maydis has been transformed and its genome sequence was released by the Broad Institute of MIT and Harvard in June 2003, updated in April 2004 (*9*) and the genomic analysis was initially reported by Kamper and collaborators (*10*) and Mewes and collaborators (*11*). With the genome sequence it has been possible to make comparative genomics to examine the genetic basis of pathogenicity, and different researches have been able to survey the inventory of predicted genes encoding components of signaling pathways, and analysis derived from a combination of genome sequence inspection and direct experimental evidence (*12*, *13*).

The Life Cycle

U. maydis has been established as a valuable model system to study fungal dimorphism and pathogenicity, and the mating reaction induces a morphogenetic switch from budding to filamentous growth (*7*, *10*, *14*). In its haploid stage, *U. maydis* is unicellular and multiplies vegetatively by budding and can be propagated in the laboratory in this form; also, it can be manipulated efficiently by reverse

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genetics and transformation. The filamentous stage appears only inside the plant cell and in specific laboratory conditions (*15*). Therefore, *U. maydis* is used as a powerful tool to analyze the relationships between cell cycle, morphogenesis, and pathogenicity (*16*).

The interactions between fungal pathogens and their plant hosts, such as the ability of fungus to penetrate into the plant and pass through several plant defense mechanisms are a very complex plant-fungi relationship. Besides, the fungus needs to maintain plant cells alive in order to ensure its survival, therefore, the plant response is attenuated (*17*, *18*).

U. maydis is a biotrophic pathogen that requires living host tissue to complete its infection cycle; it is a heterothallic fungus with a tetrapolar mating system and a dimorphic life cycle. *U. maydis* has a saprophytic asexual phase, it can grow as yeast in an asexual life cycle and also under a parasitic phase that generates sexual spores that undergo meiosis upon germination (*8*, *10*, *19*, *20*). Sexually compatible *Ustilago* cells harbor different alleles at the mating type loci *a* and *b*, and when two sexually compatible cells meet on the plant surface, they form a dikaryon filament and then enter into the sexual cycle (*8*, *19*, *21*).

U. maydis is initially recognized by the plant innate immune system and induces a non-specific response. The activation of virulence in pathogenic fungi often involves differentiation processes that need the reset of the cell cycle and induction of a new morphogenetic program. Therefore, the fungal capacity to modify its cell cycle constitutes an important determinant to catty out a successful infection. The virulence process in *U. maydis* implies strong morphological (bud to hyphae, and hyphae to teliospore transition) and genetic changes (haploid to dikaryotic, then to diploid transition). The initial recognition of the fungus on the leaf surface elicits strong and unspecific defense responses of maize plants (*22*, *23*).

In order to colonize plants with *U. maydis*, it is necessary to avoid and suppress plant defense responses and acquire nutrients rich in carbon and nitrogen, redirecting the metabolism of the host to the site of infection and manipulate the plant cell cycle to create the environment for its proliferation (*24*). Once the fungus has penetrated the host tissue, a biotrophic interface is established in which defense responses and programmed cell death are suppressed (*19*). In brief, once inside the plant, the mycelium grows and triggers the colonization of plant tissues (Figure 3). Fungal development in plant tissue is accompanied by the formation of large tumors in which plant cells divide and enlarge, presumably in response to fungal signals. Completion of the sexual cycle and teliospore (diploid spore) production occurs under normal circumstances only in the infected plant (*5*, *25*).

The first step of infection is the *U. maydis* teliospore germination (Figure 3). Meiosis leads to the production of haploid spore or sporidia that normally are not capable of causing infection alone; they are saprophytic and grow by budding (*5*, *26*, *27*). Haploid budding cells grow readily in laboratory conditions, and the role of this form in the life and disease cycle of *U. maydis* in nature is not well understood; however, sporidia produced by budding have been proposed as the primary infectious agents in nature for its exponential gowth (*28*). Teliospores as well as sporidia can be transported by wind over hundreds of miles, thus increasing the range over which *U. maydis* can survive (*2*, *5*).

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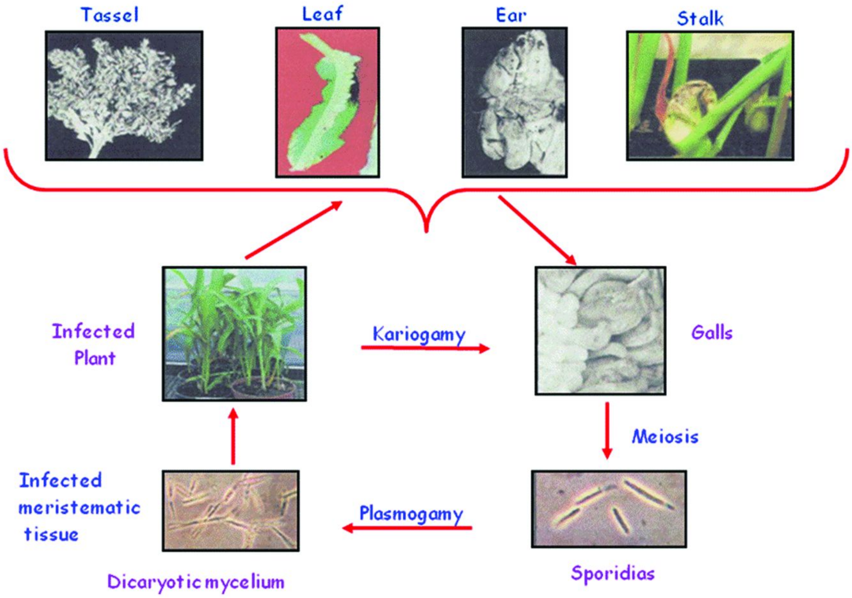

Figure 3. Ustilago maydis life cycle.

Haploid cells must fuse to form a dikaryon, which is the infecting status inside the plant. The infection is found almost exclusively on immature epidermal cells, which present low mechanical resistance. Besides, anthocyanin production is a characteristic symptom in inoculated maize seedlings (Figure 3) (*29*, *30*).

The filamentous dikaryon of *U. maydis* establishes a biotrophic interaction that persists throughout the disease cycle (Figure 3); specific signals from the host plant are apparently necessary for the maintenance and proliferation of this structure (*20*). As a biotrophic pathogen, *U. maydis* depends on the survival of colonized host cells (*23*, *31*).

Once fungal colonization is underwent, the most remarkable symptom induced by *U. maydis* is hypertrophy of plant cells or gall formation, which is observed macroscopically in the form of tumors (*29*, *32*, *33*). *U. maydis*–induced tumors are formed by enlargement and proliferation of plant cells. Large fungal aggregates are formed in tumors, and this occurs without the elicitation of programmed cell death in the surrounding plant tissue (*31*). Induction of tumor growth is also accompanied by accumulation of anthocyanins, resulting in a red pigmentation of the infected tissue (*19*, *22*, *23*).

The histology of these galls is consistent with the theory that an excess of plant hormones contributes to their development, in particular, markedly elevated indole-3-acetic acid levels (*29*, *32*–*40*). Within those tumors, a massive proliferation of diploid teliospores takes place; *U. maydis* produces up to 6 billion teliospores per cubic centimeter of gall tissue (*5*). The diploid sexual spores are only produced when *U. maydis* is growing in plant; their development also appears to be dependent on signals from the host plant. Therefore, the infectious dikaryon requires a host for proliferation and formation of sexual spores; it is not possible to reconstitute this cycle *in vitro*. Many of these steps require stage-specific signals from the host (*19*).

Genome Structure

Valverde and collaborators (*41*) demonstrated that RFLP fingerprinting is a useful method for detecting genetic variation in populations of *U. maydis*. It can be utilized as a reliable tool to discriminate populations of this fungus and to study genetic relationships. Also, they indicated that field populations of *U. maydis* are composed of genetically diverse isolates, demonstrating considerable genetic variation within field populations.

The *U. maydis* genome has been sequenced independently. Two sequencing projects were carried out in 2003 and 2004 by private companies (Bayer Crop Sciences and Excelixis, Inc.) (*42*). Later, in 2008, the Broad Institute of MIT and Harvard sequenced the wild-type strain 521 and released the information to the public (*43*). This was accomplished as part of the Whitehead Institute's Fungal Genome Initiative and the Munich Information Center for Protein Sequences (MIPS) (*U. maydis* database) (*44*).

The *U. maydis* genome has 20 Mb with approximately 6,500 genes in its 23 chromosomes (*45*, *46*). 12 gene clusters encoding secreted proteins expressed in plant during infection, have been identified in *U. maydis* genome and transcriptome, and five of these clusters are functionally involved in tumor formation. In addition, *U. maydis* has effectors with organ-specific expression, showing essential roles in tumorogenesis (*10*, *47*). Walbot and Skibbe (*48*), based on gene deletion assays and analysis of the *U. maydis* genome, explain that this pathogen secretes effector proteins that trigger abnormal host cell differentiation.

Hsiang and Baillie (*45*) and Kamper and collaborators (*10*) reported that *U. maydis* contains a strong set of genes encoding plant polysaccharide degrading enzymes that weaken the plant cell wall and allow penetration. A limited number of endoglucanases, endoxylanases, endo- and exo-acting enzymes are present. Several putative lipases and four cutinases that possibly degrade plant lipids and the maize cuticle, respectively, were detected in the *U. maydis* genome. It also contains several lignin-related activities, specifically laccases and chloroperoxidases. A large set of extracellular proteases, six putative extracellular nucleases and three phytases were detected in the genome sequence. A small number of genes encoding putative extracellular chitin and glucan enzymes were also detected, and finally, several genes encoding putative secreted metabolic enzymes were found in the *U. maydis* genome. Lipases, proteases, nucleases and phytases were found in the fungus counteracting plant defense systems, as well as the release of nutrients (*6*, *10*, *24*). Nutrients are redirected from healthy leaves of infected plants, suggesting the ability of *U. maydis* to redirect the metabolic flow of the host towards infected areas (*23*, *49*).

Böhmer and collaborators (*50*) identified protein spots with an increased intensity after dimorphic transition from budding to filamentous growth. Approximately 25% of the proteins are up-regulated exclusively by the

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b-heterodimer (*10*). The *b*-regulated secreted proteins include PEP4, glutaminase A, HOM6, and protein disulphide isomerase. Gene clusters encoding secreted proteins play a decisive role during pathogenic development in plant, but none has been limited to pathogenic development. Kamper and collaborators (*10*) clearly mentioned that they could not identify all proteins associated with filamentous growth; however, they reported 13 protein spots whose expression is significantly increased in filaments compared to budding cells. On the other hand, it is necessary to reveal a better understanding of the relationship between transcription and protein abundance (*50*).

Based on sequence comparisons, Estrada and collaborators (*51*) identified candidate genes responsible for the formation of β-carotene and retinal, and showed that *U. maydis* accumulates moderate amounts of β-carotene. Additionally, McCann and Snetselaar (*52*) reported a large number of differences between *U. maydis* and other basidiomycetes through a general genome-based analysis of amino acid metabolism, and established that this fungus could be more similar to mutualistic fungi than to many other plant pathogenic fungi.

Huitlacoche. General Aspects and Composition

Huitlacoche has been traditionally prized as a delicacy since the Aztec times, and several hundreds of tons, if not some few thousands, of huitlacoche are sold annually during July and August at markets in various regions of México, and dozens of tons in the rest of the year. It is sold fresh, but is also commercialized, canned and lyophilized, by at least six companies (Figure 4) (*2*, *53*–*55*).

Nowadays, in the USA there has been a great interest to produce huitlacoche due to an emerging acceptance by the North American public, who consideres that it as a gourmet food and can be purchased on the Internet at high prices ((*56*, *57*)*, personal observations*). The use of huitlacoche as food has spread to the point that it is currently a culinary delight for some international chefs due to the unique mixture of components that produce its flavor, aroma and organoleptic characteristics (*58*, *59*).

Lizárraga-Guerra and collaborators (*59*) demonstrated that a large number of compounds play an important role in the overall aroma of huitlacoche, such as hexanal, octanal, decanal, (*E*,*E*)-deca-2,4-dienal, (*E*)-undec-2-enal, and vanillin are the most potent odorants of huitlacoche. The fact that the aromatic compounds of huitlacoche are mainly aldehydes could lead to the oxidation of fatty acids, such as oleic or linoleic acid, which are the major fatty acids of this food. Huitlacoche contains important amounts of flavoring amino acids that might play a significant role in producing the characteristic “umami” flavor. Huitlacoche could be considered a valuable supplement for the human diet, although it is mostly eaten due to its flavor (*60*). The unique flavor of huitlacoche, as found in our laboratory, has given the basis to suggest that its characteristic flavor should be termed as “huitlacoche flavor”; in the same way as vanilla and chocolate flavors are recognized (*61*).

Table I. Proximate composition of huitlacoche (dry basis). Data are from references (*53*, *62–64*)

Compound	*Huitlacoche (g/100 g)*
Protein (N x 6.25)	9.7 - 16.4
Fat	1.6 - 3.8
Ash	4.5 - 7.0
Fiber	16.0 - 60.0
Total carbohydrates	53.3 - 81.5

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Table II. Amino acids composition in huitlacoche (dry basis). Data are from references (*53*) and (*62*)

Amino acids	*Protein (g/100 g)*
Histidine	2.0 - 2.9
Isoleucine	2.4 - 2.6
Leucine	4.6 - 5.2
Lysine	6.3 - 7.3
Methionine	2.2 - 3.2
Cysteine	2.2 - 3.7
Phenylalanine	3.7 - 4.5
Tyrosine	1.2 - 1.7
Threonine	3.4 - 3.9
Valine	3.3 - 3.9
Alanine	5.7 - 6.0
Arginine	4.1 - 4.4
Aspartic acid*	11.4 - 14.1
Glutamic acid*	15.9 - 16.9
Glycine	7.3 - 8.3
Proline	3.6 - 4.0
Serine	8.7 - 9.6

* Mainly asparagine and glutamine.

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San Miguel
Cuitlacoche
Contenido Neto / 215 g
Cuitlacoche
CUITLACOCHE
HERDEZ
Vegetales del Bajío
Cremissima
Auténtico Sazón Casero
Instant Cream Soup
Crema de HUITLACOCHE
BLACK MAIZE
CONTIENE LECHE Y MANTEQUILLA
CONTAINS MILK AND BUTTER

Figure 4. Canned and lyophilized samples of huitlacoche available in the market.

The evaluation of total proteins, amino acids, dietary fiber, carbohydrates, unsaturated fatty acids and other components has demonstrated that huitlacoche contains interesting nutrimental and bioactive substances, consequently, this fungus may be considered a nutraceutical food. Valverde (*53*) and Valverde and Paredes-Lopez (*62*) reported the proximate composition and fatty acids profiles of huitlacoche collected from two different locations where it naturally grows, 15 creole maize cultivated in El Bajío, México and one hybrid maize cultivated in Urbana, IL (*63*, *64*). The protein content ranged from 9.7 to 16.4% (dry basis) (Table I), sometimes is superior to other edible mushrooms and definitely higher than the maize protein content (10%). Therefore, huitlacoche could be proposed as an alternative protein source for vegetarian diets as other edible mushrooms have been suggested (*61*, *65*). Huitlacoche contains almost all essential amino acids (Table II). Lysine (6.3 to 7.3 g/100 g protein) is one of the most abundant amino acids. Other abundant amino acids are aspartic and glutamic acid, glycine and serine, which collectively accounted for 49.6 to 56.2% of the total amino acids in the samples (*53*, *62*). Lysine is a limiting amino acid in the nutritional value of corn, but huitlacoche is one of the foods with the greatest content of this amino acid (*61*).

Most of the total fatty acids are oleic and linoleic acids (54.5 to 77.5%) (Table III). The high content of essential fatty acids also suggests that huitlcoche has an interesting nutritional value, and it may be due to the fact that corn is one of the cereals with the greatest content of fats and with a good proportion of essential unsaturated fatty acids as well (*53*, *62*, *63*, *66*).

Another important nutritional factor of a food product is the content and type of carbohydrates, Valdez-Morales (*63*) and Valdez-Morales and collaborators (*64*) reported eight monosaccharides and eight alditols in huitlacoche. Two monosacharides, glucose and fructose, were the most abundant carbohydrates. Arabinose, galactose, manose and xilose were found in less proportions (Table IV). The relatively high content of glucose and fructose is another remarkable aspect of this fungus.

Table III. Fatty acids composition of huitlacoche. Data are from references (*53*), (*62*), and (*63*)

Fatty acids	*Total content (%)*
Palmitic (C16:0)	13.0 - 19.0
Palmitoleic (C16:1)	2.8 - 3.8
Stearic (C18:0)	2.4 - 7.7
Oleic (C18:1)	25.2 - 29.1
Linoleic (C18:2)	29.3 - 48.4
Linolenic (C18:3)	2.5 - 2.9
Araquidonic (C20:0)	1.3 - 8.2
Others	6.6 - 8.1

Glycerol, glucitol and mannitol were the most representative alditols. On the other hand, disaccharides like trehalose and sucrose were not detected but homoglycans and heteroglycans were found, these compounds are part of the dietary fiber (*63*, *64*). The content of total dietary fiber, soluble dietary fiber and insoluble dietary fiber are higher in huitlacoche than in corn (Table IV) (*63*, *64*).

Table IV. Dietary fiber fractions, β-glucans and free sugars in huitlacoche (dry basis). Data are from references (*63*) and (*64*)

Component	***Units***
	Total content (%)
Total dietary fiber	**39 - 60**
Soluble	**9 - 29**
Insoluble	**22 - 51**
	Huitlacoche (mg/g)
β-glucans	**20 - 120**
Monosacharides	**450 - 779**
	Huitlacoche (mg/g)
Total free sugars	**56 - 267**
Glucose	**53 - 231**
Fructose	**19 - 138**
Galactose	**0.2 - 3.5**
Arabinose	**0.2 - 3.3**
Mannose	**0 - 1.8**
Xylose	**0 - 2**

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β-glucans are water-soluble or insoluble polysaccharides with a large variety of structures, and possess antitumor and immunostimulating properties. In addition, β-glucans improve the response of the macrophages and killer cells, and are the main compounds in edible mushrooms that confer anticarcinogenic activity (*66–68*). They can also be anti-oncogenic due to their protecting effect against genotoxic compounds, and their anti-angiogenic effect (1→3) backbone and (1→6) branch points are the most known antitumor structures (*69*, *70*). The content of β-glucans in huitlacoche is higher than that reported in corn and similar to other edible mushrooms (Table IV) (*63*, *64*, *71–73*).

Valdez-Morales (*63*) and Valdez-Morales and collaborators (*64*) reported antimutagenic capacity (41 to 76%) in huitlacoche, but they did not assess the compounds involved in this activity. Besides, the antioxidant activity of the phenolic compounds is related to the antitumoral activity. Valdez-Morales (*63*) also concluded that the total phenol concentration in huitlacoche is high and comparable to that reported for other edible fungi (Table V).

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Table V. Phenolic compounds in huitlacoche from creole Mexican maize (dry basis). (Adapted with permission from reference (*63*). Copyright 2010)

Phenolic compound	*Huitlacoche (μg/g)*
Gallic acid	2.4 - 2.6
Ferulic acid	514.1 - 544.2
Caffeic acid	26.3 - 27.4
p-Coumaric acid	10.2 - 10.6
o-Coumaric acid	4.4 - 4.8
Rutin	6.2 - 6.4
Catechin	11.0 - 11.7
Quercetin	42.4 - 45.2
Total phenols	636.8 - 667.4

*HPLC assay in huitlacoche methanolic extracts.

On the other hand, mycotoxins like aflatoxins, ochratoxins and zearalenone have not been described in huitlacoche (*53*, *62*); in addition, Serafín-Muñoz and collaborators (*74*) found relatively low concentrations of metals (Cr, Cu, Fe, Mn, Ni, Pb).

In recent years, some scientists have tried to find specific natural bioactive substances to improve human health or reduce diseases. In this context, huitlacoche has been characterized as an interesting nutraceutical food, as well as an attractive ingredient to enrich other foods (*3*). It must be incorporated into the daily diet because of its attractive characteristics, such as a unique flavor, and bioactive compounds. Mushrooms are valuable health foods with long recognized nutritional value, and their production under different conditions may improve the concentration of selected nutrients and compounds with therapeutical potential.

Huitlacoche Production

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Huitlacoche is sold raw, in prepared foods or processed in Mexican local markets; however, the introduction of this food into the international market in countries like USA, Japan, China and some of the European Community, as France, Spain and Germany, requires the development of techniques for massive production during the whole year.

An efficient method to inoculate maize plants with *U. maydis* was used in the 18th century to demonstrate the causal relationship between common smut and *U. maydis* (*5*). Later, Walter (*75*) and Christensen (*5*) tested different methods for inoculating maize with *U. maydis*, most of which induced stalk, leaf, and tassel with galls of common smut. Studies from the past two decades have focused on ear infection, one of the most important data is that the silk-channel inoculation procedure results in a higher incidence of ear galls than natural infection (*1*, *54*). Nowadays, it is known that ear galls can be induced effectively by injecting a sporidial suspension into the silk channel or through husk leaves as soon as silks emerge, these inoculation methods can be used to produce huitlacoche (Figure 5); sweet corn is the most susceptible for infection (*1*, *4*, *30*, *53*, *62–64*, *76–80*).

The factors that affect the yield of huitlacoche are the *U. maydis* strains, the efficiency in the production of the inoculum, the timing of inoculation and harvest, and the characteristics of the corn hybrid (*1*, *53*, *54*, *57*). The inoculum must be generated in a controlled process with two sexually compatible strains of *U. maydis* cultivated separately in potato dextrose broth (PDB), under constant stirring at room temperature for 18 to 24 h. For plant inoculation, each strain is adjusted to a concentration of 10^6 cells/ml of medium and mixed, then the mixture is injected in the silk-channels of the corn or in the leaves once the silk has emerged (*1*, *53*, *54*, *57*). Field studies suggest that the infection is more severe when the plants are inoculated between 4-8 days after silk emergence. This short period is when maize ears are susceptible to infection by *U. maydis*, and the incidence of infection (gall formation) decreases rapidly with silk age, and also when silks are exposed to pollen (*1*, *54*, *57*, *81*, *82*). When the first pollen tube reaches a maize ovary, an abscission zone of collapsed cells are formed from disorganized tissue at the base of the silk. Maize kernels seems to be protected from infection by *U. maydis* after the formation of the abscission zone because *U. maydis* infection filaments are unable to grow through this layer of dead cells (*57*, *83*). About one week after the silks emerge from ear shoots, tissues at the base of the silk begin to collapse due to senescence (*84*, *85*). Consequently, the efficient production of huitlacoche by inoculating silks with *U. maydis* requires accurate timing of inoculation and control of pollination to maximize the number of kernels infected (*i.e.*, number of infected ovaries) and yield of huitlacoche (*57*).

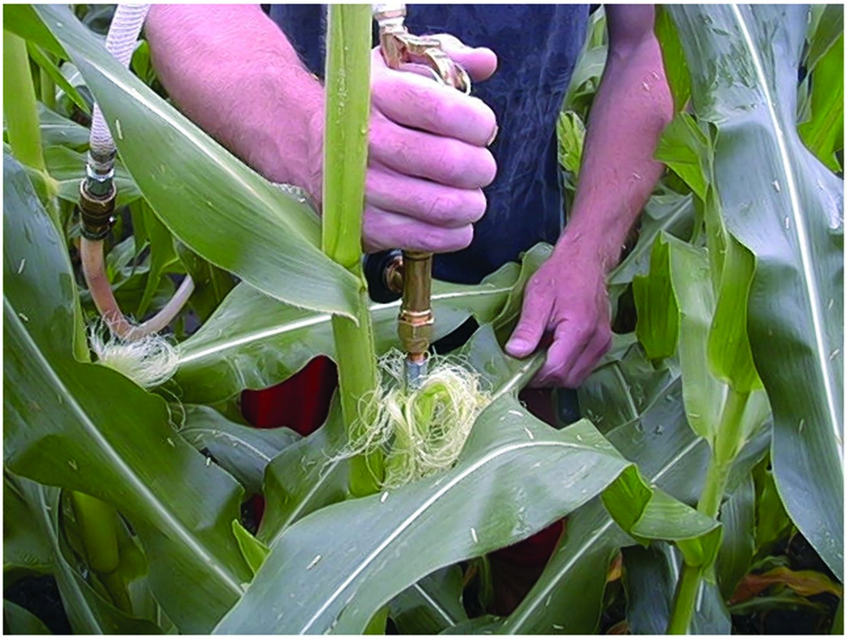

Figure 5. Inoculation method for huitlacoche production.

Pataky and Chandler (*57*) reported that the yield of huitlacoche is affected by inoculation time, pollination treatments and an interaction between these independent variables. In conclusion, huitlacoche production appears to be maximized when silks of unpollinated, large-eared field corn cultivars are inoculated with sporidia of *U. maydis* about five days after silk emergence. Optimal yield of high quality huitlacoche occurs about 16-17 days after inoculation (*1*, *53*, *54*, *57*). The taste, aroma and nutritional value of huitlacoche are dependent of the variety of corn and the state of development in which the fungus is harvested (*63*, *64*).

Postharvest

Valverde and collaborators (*1*) reported that the gall weight increased 250-500% between 14 and 21 days after inoculation, reaching 280-600 g per corn cob. Gall tissue was nearly 100% black and lost its spongy integrity 19 to 22 days after inoculation, when powdery teliospores appear (Figure 6). The optimal huitlacoche harvest time varied, among hybrids, from 5 to 24 days after inoculation depending on the maize hybrid, the growth stage at which the

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host was inoculated, and the environmental conditions. Authors concluded that huitlacoche has a narrow window of harvest time to optimize yield and quality.

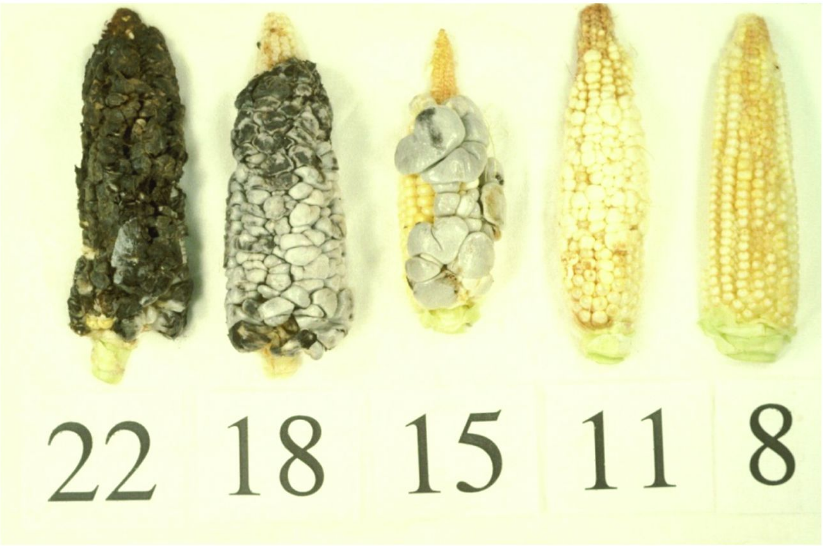

Figure 6. Huitlacoche development at various stages.

Farmers know by experience that huitlacoche keeps, in an optimal way, its appearance, color, texture and sensory characteristics when is left on the ear and especially when is stored at low temperatures. Martínez-Flores and collaborators (*86*) reported that the average respiration rate of huitlacoche on the ear at room temperature, and at 10 and 3 °C is 320.8, 120.8 and 71.5 ml $CO_2 \cdot kg^{-1} \cdot h^{-1}$, respectively; but when is cut off from the ear this rate increased considerably (372.8, 346.7 and 164.1 ml $CO_2 \cdot kg^{-1} \cdot h^{-1}$, respectively).

Final Considerations

Approximately 2,000 species of edible mushrooms are known by the societies of the world; however, no more than 20 species are commercially cultivated (*61*).

Mushrooms have been mainly present in the diet due to their sensory properties, especially by their flavor. Additionally, it has been found in the last decades that most of the fungi available in the market possess health-promoting compounds such as biologically-active polysaccharides, peptides, lipids, phenols, triterpenes, cerebrosides, and others (*87*).

The commercial importance of huitlacoche have been rised in selected markets of some countries, besides México. We have developed a technical procedure for its massive production. Because of its unique flavor, we have suggested to recognize that there is a huitlacoche flavor in the same way that there are well-known flavors such as vanilla or chocolate. (*61*).

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Interestingly, huitlacoche is relatively rich in lysine, when maize is not, and most fatty acids are represented by linoleic and linolenic acids. On the other hand, it is known that dietary fiber absorbs hazardous materials such as carcinogenic substances, inhibiting their absorption in the intestine. Glucans have also various health-related functions; these components, among others, are contained in remarkable quantities in huitlacoche (*64*).

In brief, although further studies are pending, it may be stated that huitlacoche is a valuable health food and a culinary delicacy; its consumption, in various presentations, will most likely be increased in the near future.

Acknowledgments

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Chapter 8

Opuntia spp. as a Source of Bioactive Compounds

Marizel G. Astello-García,[1] María del Socorro Santos Díaz,[2] Antonio Reyes-Agüero,[3] and Ana Paulina Barba de la Rosa*,[1]

[1]IPICyT, Instituto Potosino de Investigación Científica y Tecnológica A.C., Camino a la Presa San José No. 2055, Lomas 4ª Sección, 78216 San Luis Potosí, S.L.P., México
[2]Facultad de Ciencias Química, Universidad Autónoma de San Luis Potosí, 78210 San Luis Potosí, S.L.P., México
[3]Instituto de Zonas Desérticas, Universidad Autónoma de San Luis Potosí, 78210 San Luis Potosí, S.L.P., México
***E-mail: apbarba@ipicyt.edu.mx**

The genus *Opuntia* represents an important food and feed resource and its economic importance has gradually increased worldwide as a health-promoting food. *Opuntia spp.* contains a number of potentially active nutrients and bioactive compounds with different therapeutic uses. Wild *Opuntia* species are a source of novel metabolites and its cell culture system represents a potential renewable source of those compounds.

Introduction

The cactus pear (*Opuntia spp.*) represents the most symbolic native plant in Mexico that has been used since prehispanic times as documented in the Florentine Codex (Fig. 1). *Opuntia* species as well as maize, amaranth and agave were important in the ancient agricultural economy and basic components of the diet (*1*).

The semiarid region of the central part of Mexico hosts the greatest diversity of this cactus in the world (*2*). The *Opuntia* genus includes 188 species, and 78 are native to Mexico (*3*). Most of wild and cultivated species are located in the Central and High Plateau zones distributed in an area of three million hectares (*4*). The most abundant wild species are *Opuntia streptacantha* Lem., *Opuntia leucotricha*

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DC., *Opuntia robusta* H.L. Wendl., *Opuntia cantabrigiensis* Lynch, *O. rastrera* Weber, *Opuntia hyptiacantha* and *Opuntia chavena* (*5*). The cultivated varieties belongs to the domesticated *Opuntia ficus-indica* L. This species is well adapted to extreme climate and edaphic conditions, growing in dry, hot climates of northern Mexico, southwestern United States, Africa, Mediterranean countries and Europe.

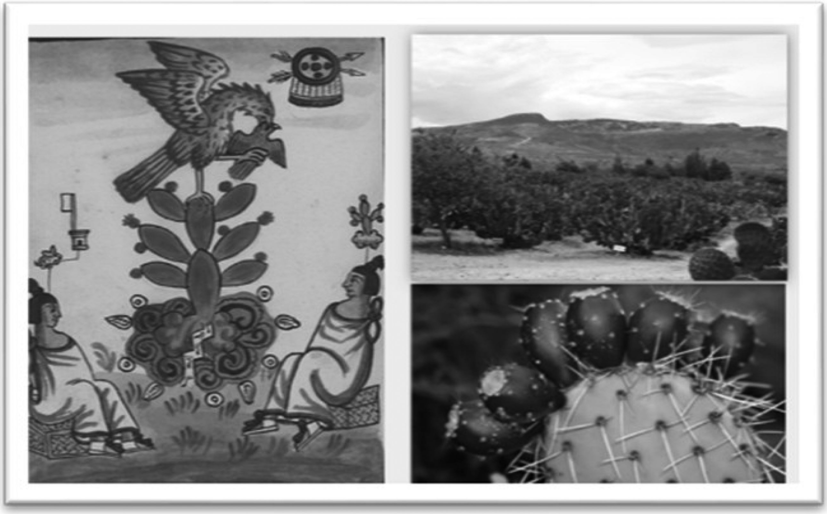

Figure 1. The Aztecs, led by the prophecies of Huitzilopochtli (god of sun and war), ended their migration through the construction of Tenochtitlan on an island in a lake where an eagle with a snake perched on a cactus in bloom.

Opuntia plants produce edible stems known as pads, vegetable, cladodes, nopales or pencas. The tender young part of the cactus stem, young cladode or "nopalito", is frequently consumed as a vegetable in salads, while the cactus pear fruit is consumed as a fresh fruit. In Mexico, 10,200 ha of *Opuntia spp*. are cultivated for production of nopalito and 51,112 ha for cactus pear. Under optima conditions annual production can reach 50 tons of dry matter per hectare (*6*).

Cladodes Chemical Composition

Opuntia spp. have a high nutritional value, mainly due to their mineral, protein, dietary fiber and phytochemical contents (*7*). The main constituent of *O. ficus-indica* cladodes is water (80-95%), followed by carbohydrates (3-7%), fiber (1-2%), and protein (0.5 – 1%). However, as shown in Table I, the chemical composition on dry weight of commercial and wild species depends on variety, maturation stage, environmental conditions and manufacturing techniques (*1*, *8*).

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The carbohydrate fraction includes mucilaginous components containing polymers, such as chains of (1-4)-linked β-D-galactouronic acid and α(1-2)-linked L-rhamnose residues (*9*). Ginestra et al. (*10*) reported that glucose and galactouronic acid were the main sugars of *Opuntia* cladodes.

Table I. Proximate composition of different commercial and wild *Opuntia* spp. (*adapted with permission from reference* (*8*))

Variety	*Component (%)*				
	Protein	*Fat*	*Fiber*	*Ash*	*CH*
Blanco[1]	6.7	0.1	15.0	17.3	61.4
Amarillo[2]	15.1	0.6	6.2	15.9	63.2
Cristalino[2]	9.4	1.5	7.7	14.8	66.5
Duraznillo[2]	13.5	1.1	7.1	19.7	69.8
Tapon II[2]	17.4	1.8	20.4	19.5	42.4
Tempranillo[2]	13.4	nd	5.5	19.3	61.9
Tablets[3]	4.2	0	51.6	37.6	6.7

CH=Carbohydrates, nd=not detected, 1=domesticated species, 2=wild species, 3=product found at commodity market.

Therapeutic Uses

Besides being a traditional source of vegetable, cladodes also have medical applications. The *O. ficus-indica* var. *saboten* is used for wounds, burns, edema, dyspepsia, and as a neuroprotective, cytoprotective, antispasmodic, and chemopreventive in traditional medicine (*11–16*). The cladode of the plant is traditionally used to treat asthma, gastritis, intestinal colic and ulcers (*17*). The extracts of fruits and stems exhibit hypoglycaemic (*18*, *19*), anti-allergic (*20*), antioxidant (*21–24*), and anti-inflammatory (*25*) activities.

Table II shows a brief summary of some uses and the molecule that is claimed as responsible .

Clinical studies have shown the effect of nopal on type 2 diabetic patients (*26–31*), suggesting that the the anti-hyperglycemic effect may be due to its fiber and pectin content, which may decrease carbohydrate absorption (*32*). On the other hand, it was confirmed that the plant and filter plant extracts from *O. streptacantha* produce the anti-hyperglycemic effect on streptozotocin (STZ)-diabetic rats, and some other bioactive compounds more than fiber and mucilage are responsible of this beneficial activity (*31*). Also it was demonstrated that the petroleum ether extract from the edible part of *O. Milpa Alta* showed remarkable decrease of blood glucose levels, which may be a potential natural hypoglycaemic functional ingredient (*33*).

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Table II. Therapeutic effecs of *Opuntia spp.*

Species	***Effect***	***Extract***	***Metabolite***	***Model***	***Ref***
O. ficus-indica var Mill.	Healing	Methanolic	β-sitosterol	Male ICR mice	(*12*)
O. ficus-indica var Mill.	Cytoprotective	Mucilage and pectin fractions	Mucilages, pectins	Male Wistar rats	(*13*)
O. ficus-indica var Mill.	Neuroprotective	Ethyl acetate fraction	Flavonoids	Cortical cell cultures (from Sprague–Dawley rats)	(*14*)
O. ficus-indica var Mill.	Antispasmodic	Fresh juice	Indicaxanthin	Adult male mice (C57BL/10SnJ)	(*15*)
O. ficus-indica var Mill.	Chemopreventive	Raw cladode	---	Balb/c male mice	(*16*)
O. robusta	Hypocholesterolemic Antioxidative	Broiled cladode	---	Humans with hypercholesterolemia	(*21*)
O. ficus-indica var Mill.	Antioxidant Antigenotoxic	10 mM Tris–HCl homogenated	---	Balb/c mice	(*22*)
O. ficus-indica var Mill.	Antioxidant and antiulcerogenic	Juice	Flavonoids	Male Wistar rats	(*23*)
O. ficus indica f. inermis	Antioxidant and antiulcerogenic	Methanolic	Phenolics, flavonoids, polysaccharides	Male Wistar albino rats	(*24*)
O. ficus-indica var Mill.	Hypoglycemic	Raw cladode	---	STZ-induced diabetic rats	(*26*)
O. monacantha	Hypoglycemic	Raw cladode	Polysaccharides	STZ-induced diabetic rats	(*27*)
O. streptacantha	Hypoglycemic	Fresh juice	Secondary metabolites	New Zealand adult male rabbits	(*28*)
O. ficus-indica var Mill.	Hypoglycemic	Raw cladode/ fruit	---	Non-diabetic Wistar rats	(*29*)

Continued on next page.

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Table II. (Continued). Therapeutic effecs of *Opuntia spp.*

Species	*Effect*	*Extract*	*Metabolite*	*Model*	*Ref*
O. dillenii	Anti-diabetic	Total polysaccharide extract	Polysaccharide (ODP)-Ia	STZ-induced diabetic mice	(*30*)
O. Milpa Alta	Hypoglycemic.	Petroleum ether fraction	---	STZ-induced diabetic rats	(*32*)
O. humifusa	G1 arrest and proliferation inhibition	Aqueous fraction	---	Glioblastoma-human cancer cell line (U87MG)	(*42*)

A recent study by Godard et al. (*34*) has shown that the commercial preparation made from cladode extracts and fruit skins of *O. ficus-indica* (OpunDia™), was useful to decrease blood glucose. Other species such as *O. streptacantha* cladodes have been used as anti-diabetic foods (*35*). Theories such as the stimulation in the insulin secretion observed in *O. ficus-indica* (*36*) should also applied to *O. streptacantha*; but further studies should be done toward the understanding of the molecular mechanisms of *Opuntia* extracts towards its anti-diabetic effects.

Also, it was observed that *Opuntia* extracts have anti-tumoral (*37*) and hepatoprotective effects in mice (*18*). Park et al. (*38*) reported that the β-sitosterol as an active anti-inflammatory principle from the stem extract. The n-butanolic extracts of *O. ficus-indica* has beneficial effects on memory performance in mice (*39*). It was demonstrated that two flavonoids (kaempferol and quercitin), isolated from the *O. ficus-indica* var. *saboten* showed a potent antidepressant effect (*40*). An extract of the cactus plant *O. streptacantha* was able to inhibit the intracellular virus replication and to inactivate extracellular virus, but in that work, the active inhibitory components of the extract appeared to be protein in nature (*41*). The hexane, ethyl acetate, and water partitioned extracts from *O. humifusa*, were tested on proliferation, G1 arrest and apoptosis in U87MG human glioblastoma cells (*42*). In addition, *O. ficus-indica* fruits extracts were able to increase apoptosis, and to inhibit the ovarian cancer and vesicle, cervical and immortal epithelial cell growth. The inhibition has been shown to be dose-time dependent (*36*).

Phenolic and Flavonoids Present in *Opuntia spp.*

The use of natural phytochemicals present in fruits, vegetables, and herbs as antioxidants has increased. *Opuntia* seems to be a good source of those compounds (*43*). For this, the interest in the characterization of phytochemicals present in cladodes and fruits has also increased, but most of the studies have been carried out using the *O. ficus-indica* commercial samples. Few studies have been focused on the characterization and quantification of phytochemicals using wild compounds.

Medina-Torres et al. (*44*) reported that concentrations of six phenolic acids (Fig. 2A) and five flavonoids (Fig. 2B) in *Opuntia* samples varies depending on the species. Also, it was observed that processing affected the concentration; drying temperature of 45°C and an air flow rate of 3 m s^{-1} were the best conditions for bioactive compound preservation in Opuntia samples.

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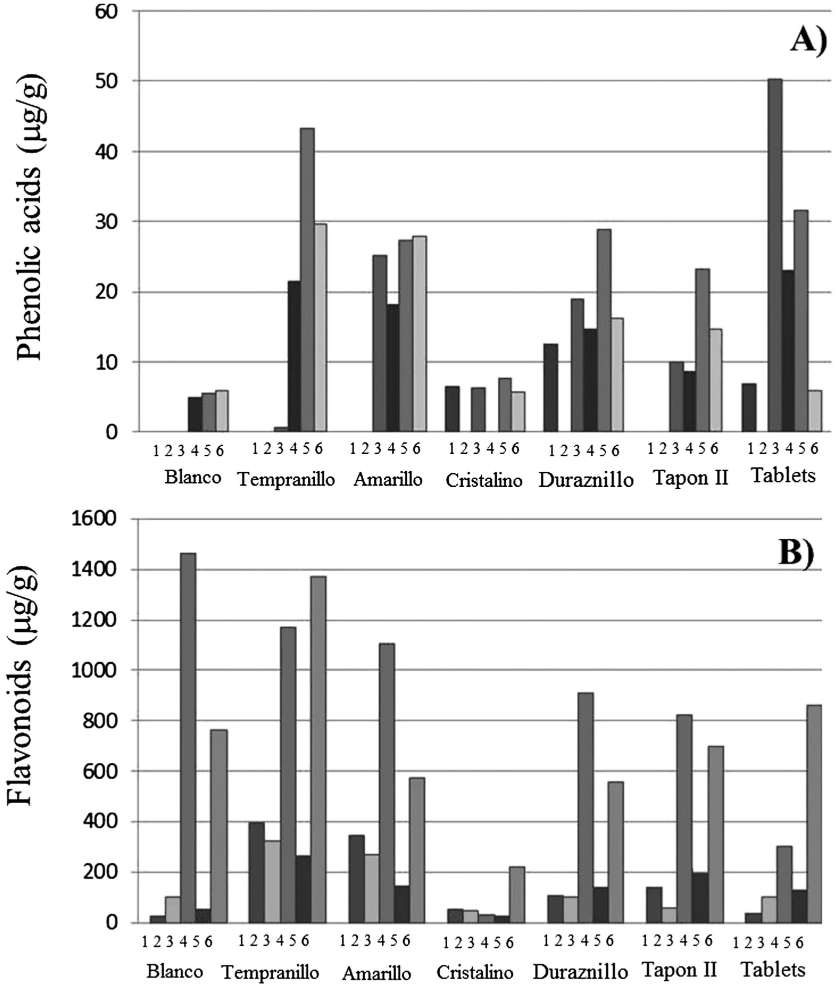

Figure 2. A) Phenolic acids: 1=gallic, 2=coumaric, 3=3,4-dihydroxybenzoic, 4=4-hydroxybenzoic, 5=ferulic, 6= salicylic. B) Flavonoids: 1=isoquercitrin, 2=isorharmnetin, 3-O-glucoside, 3=nicotiflorin, 4=rutin, 5=narcissin. (Adapted with permission from reference (8).)

Some other works has focused on the characterization of novel compounds. Two alkaloids (indicaxanthin and neobetanin) and few flavonoids were isolated (*45–47*). Moreover, the isorhamnetin-3-*O*-(6”-*O-E*-feruloyl)-neohesperidoside, (6R)-9,10-dihydroxy-4,7-megastigmadien-3-one-9-*O*-β-D-glucopyranoside and (6S)-9,10-dihydroxy-4,7-megastigmadien-3-one-9-O-β-D-glucopyranoside were isolated from methanolic extract of *O. ficus-indica* var. *saboten* (*48*). Luo et al. (*49*) analyzed the petroleum ether extract of *O. Milpa Alta* by GC/MS, and reported the isolation of 26 compounds. The most abundant was phytosterol (30%), followed by polyunsaturated fatty acids (18.6%), phytol (12.1%), palmitic acid and palmitate (13.5%), vitamin E (4.5%) and other compounds (7.5%).

Cell Culture Systems for Production of Secondary Metabolites

Plants are an important source of new compounds of medical importance for drug development. Today several distinct biomolecules, mainly secondary metabolites (alkaloids, terpenoids, steroids, saponins, phenolic, flavonoids) and amino acids derived from plants are important drugs that are currently used in one or more countries in the world (*50*, *51*). Plant cell culture systems represent a potential renewable source of valuable medicinal, flavors, essences and colorants that cannot be produced by microbial cells or chemical syntheses (*50*). The isolation of the phytochemical can be fast and efficient, when compared with extraction from complex whole plants. Thus, plant cell cultures represents an excellent biotechnological technique in production of secondary metabolites with therapeutical applications (*52*).

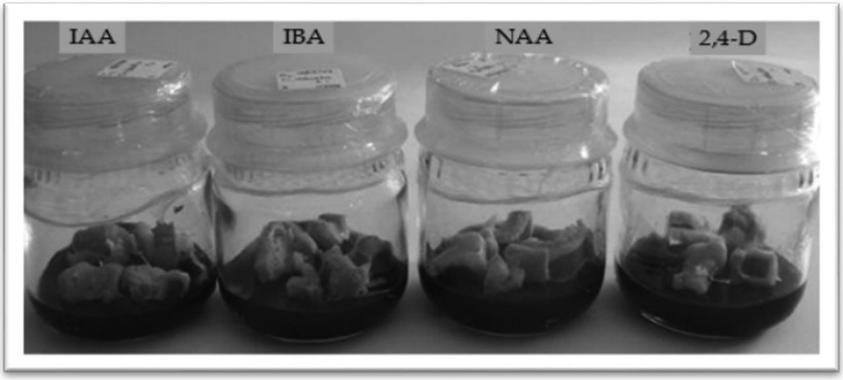

Figure 3. Callus systems from O. robusta. IAA= indole-3-acetic acid, IBA=indole-3-butyric acid, NAA= naphthalene acetic acid, 2,4-D=2,4-dichlorophenoxyacetic acid. (Reproduced with permission from reference (53).)

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A protocol for the establishment of callus cultures from non-domesticated species *O.robusta* was developed (*53*). In this work, different disinfection procedures, type of plant growth regulators (Fig. 3), and culture media composition on callus development were evaluated. *O. robusta* callus induction was achieved on MS (Murashige and Skoog) medium containing 3 mg l^{-1} indol acetic acid, 1 mg l^{-1} picloram, and 1.2 mg l^{-1} kinetin. Addition of 0.4 g l^{-1} casein hydrolysate and 0.1 mg l^{-1} biotin to the medium improved callus formation.

Accumulation of metabolites often occurs in plants subjected to stresses including various elicitors or signal molecules. It is well known that jasmonic acid (JA) and methyl jasmonate (MeJ) are signal molecules in biotic and abiotic stresses. Chitosan has been used to promoted the production of pigments in cactus *in vitro* cultures (*54*), taxol in *Taxuschinensis* (*55*), and phenolic acids in *Vitisvinifera* cell suspension cultures (*56*).

The effect of jasmonic acid and chitosan on phenoliccompound content in *O. robusta* cultures was also evaluated. The exposure of *O. robusta* callus to jasmonic acid increased 1.3-fold and 3-fold total phenolic acids and flavonoids concentration, respectively. Addition of chitosan to *O. robusta* cellular suspensions, also improve accumulation of total phenolic acids by 2.2 folds and flavonoids by 1.7 folds (Fig. 4).

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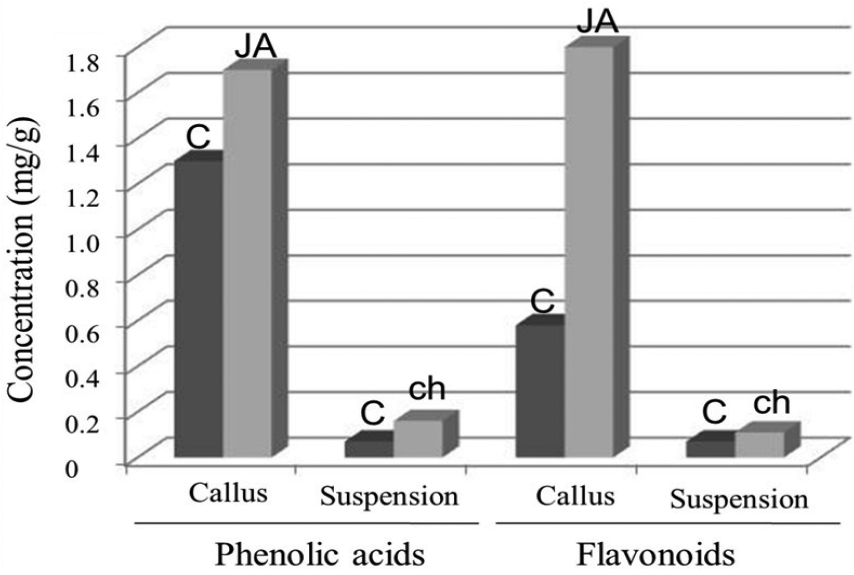

Figure 4. In vitro cultures production of phenolic acids and flavonoids. C=control samples, JA=induction with Jasmonic Acid, ch= induction with chitosan. (Reproduced with permission from reference (47).)

Conclusion

Opuntia spp. is a source of novel bioactive compounds that have a wide range of biological functions, among them the anti-diabetic effects are the most important. To date it is not clear which compounds are responsible for the observed effects, and further work must be done in order to get more insights about the molecular mechanisms of the action of *Opuntia* extracts. *In vitro* cell culture has been established and work must be done towards establishing a large-scale bioreactor process for the production of valuable nopal bioactive compounds that could have different therapeutic uses.

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Chapter 9

Nopal: A Perspective View on Its Nutraceutical Potential

Paola I. Angulo-Bejarano and Octavio Paredes-López*

**Centro de Investigación y de Estudios Avanzados–IPN, Unidad Irapuato,
Km 9.6 Libr. Norte Carr. Irapuato-León, Apdo. Postal 629, 36821 Irapuato,
Guanajuato, México
*E-mail: oparedes@ira.cinvestav.mx**

Current knowledge on *Opuntia* spp is increasing. Many research groups around the world are gaining insight in different aspects of the potential use of these plants. Much of this research is focusing mainly on nutraceutical properties in the search for new food-medical alternatives, since most of the current medicines are costly and encounter many secondary effects. Nucleic acid isolation techniques are very useful for genetic and evolutionary research, transgene integration patterns (DNA isolation), cDNA library construction (total RNA isolation), functional analysis (proteomics), and recently microRNA machinery (small RNA isolation). In addition, interest on plant cell tissue culture and genetic transformation techniques has emerged over the past decades which can lead to very powerful procedures for functional analysis or nutraceutical compounds overexpression. Finally, food industry research has focused lately on the use of natural products. *Opuntia* spp food ingredients such as dietary fiber and pigments can be incorporated into food industry formulations. Therefore, the aim of the present chapter is to summarize and contribute to the understanding of newly developed research concerning this ancient but not less important plant.

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Introduction

Among pre-Hispanic foods used by the first human settlements in the vast majority of the American continent, nopal figures as one of the main plants, along with corn, beans and maguey. Evidence such as that presented in the Codex Mendoza (1535-1550) depicts Aztec tribute indicating the use of nopal as a trade item (*1*). This codex also includes a representation of Opuntia cladodes amongst other items such as ocelot and jaguar skins. One of the main uses of this plant in Pre-Columbian times was cochineal dye production; this product also served as a tribute to the Aztecs (*1*). This ancient use for Opuntia contributed in a great way to its dispersion around the globe, and its colonization as a plague in some parts of the world. Nowadays, it is considered the most economically important cactus for its use as food and forage, as well as increasing source for nutraceuticals. In this sense, *Opuntia* spp plants are rich in these compounds as it will be further described in this chapter. Medical or nutraceutical applications include its role as a potent antidiabetic agent, which traditionally was considered a result of high mucilage, pectin and dietary fiber content; however, today some studies are not in agreement with this since they state that this effect may also be due to non-identified compound(s) present in fractions devoid of fiber, or to high mineral contents in some cultivars (*2*). Another potential use of this plant is in cancer chemoprevention as revealed by various studies (*3*, *4*). This effect has been attributed to the high antioxidant compounds present in cladodes and fruits, such as phenolic acids and polyphenols. Other nutraceutical properties such as improving long term memory and helping against allergies are also described.

Nopal characterization with the proper genetic and evolutionary tools may help to understand and preserve the genetic diversity (*5*). Therefore, the development of proper nucleic acids extraction protocols is of high importance. Some of the most important and recent works on this matter are described. Also, some total RNA isolation techniques that are currently being used for cDNA synthesis as well as for genomic library productions are described. In addition, in the era of interfering RNA and silencing machinery a new report is described which demonstrates the feasibility for small RNA isolation and its further use in other downstream applications (*6*).

One of the most important features of *Opuntia* spp plants is that they have evolved a very efficient asexual reproduction system, since many of their seeds are abortive, and present a highly lignified seed coat making very difficult the germination process. This natural asexual reproduction system along with the plant cell totipotency have both been taken and utilized in different *in vitro* plant cell cultures that are described in this chapter; many of them have proved to be successful, others are still in their way. The main intention of the implementation of these techniques in nopal is firstly, to ensure their survival, and secondly, to use these techniques in further plant genetic transformation events. For the latter aspect various approaches that have been developed in the past years are described (*7*, *8*).

In the last decades, some sectors of the population worldwide have turned back to “natural foods” which has led them to the search for new healthier alternatives, such as organic products or nutraceutical containing foods, thereby imposing a challenge for modern food industry. In this sense *Opuntia* spp plant ingredients have very much to offer such as high antioxidant compounds and dietary fiber and in the case of its fruits, antioxidants and natural pigments. The potential applications of nopal ingredients such as dietary fiber in food formulations are described as well as fruit natural pigments and their microencapsulation for the use in beverages.

History and Evolution

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Nopal (*O. ficus-indica*) constitutes the cactus species with the highest economic importance worldwide. It is grown in America, Africa, Asia, Europe and Oceania (*9*). In Mexico, it is a plant typically found in the landscape and constitutes one of the most important symbols of this nationality (*10*). Along with corn (*Zea mays,* L.), beans (*Phaseolus vulgaris*, L.) and maguey (*Agave americana*) it was a fundamental food and responsible in great measure for human settlements and cultural development of the Chichimeca groups in the center and north of the country (*10*).

It is generally considered that this species was domesticated in Mexico (*11*, *12*) where the highest richness of traditional cultivars is found (*10*). Since wild plants have not been found it has been proposed that it derived from *O. amyclaea* or *O. megacantha*, two species distributed in north central Mexico considering the fact that the most extreme degree of domestication in any given species is characterized by its dependence on manmade habitats to survive; in this sense all *O. ficus-indica* cultivars are located in protected environments, either plantations or home gardens, since their survival in areas exposed to herbivore vertebrates is unlikely (*9*). Out of its 377 recognized species, 104 are found in the wilderness of Mexico and from them 60 are endemic (*10*). The evolution of this plant in arid and semiarid environments, where the different environmental scenarios have imposed limits to the survival and productivity of desert plants, has conducted to the development of adaptation characteristics in its anatomy, morphology and physiology; a process that has been poorly studied, yet there are some interesting findings in this respect. In this sense, an early work proposed that *O. ficus-indica* was a spineless cultivar derived from *O. megacantha,* a species of central Mexico (*13*). However, some authors (*14*) explored this relationship with amplified fragment length polymorphism (AFLP) data and reported that *O. ficus-indica* had the closest affinity with *O. megacantha*. According to Uphof (*15*) and Zeven and Zhukovsky (*16*) the origin of this cactus is within Mexico, but without reference to a specific region. The taxonomical concept of *O. ficus-indica* is somewhat uncertain. This species is sometimes described as spineless and taxonomically distinct from other cultivated opuntias, such as *O. megacantha, O. streptacantha* and *O. amyclaea* (*9*). In other cases spined, cultivated genotypes have been classified as *O. ficus-indica* (*17–19*). Actually, the presence of spines in the cladodes is an inadequate feature to discriminate *O. ficus-indica* from other arborescent opuntias (*19*, *20*). Within the genus, the growth habit, the presence of spines, the number of spines per areole, and the number of areoles may differ drastically in different growing regions (*21*). Also, several authors reported that

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it is difficult to correctly assign cultivated genotypes to a defined taxon (*14*, *20*, *22*). The continuous morphological variation within the genus, the lack of clear descriptors for each species, and the relative ease of cross hybridization (with the presence of individuals with intermediate characteristics) have led to an erroneous species designation. As a result of this, the same cultivars are often classified as belonging to different species, and in other cases they are considered to be hybrids among unknown parental (*12*).

The history of nopal has been little studied (except that of the cochineal). Linnaeus classified *Cactus opuntia* and *C. ficus indica* in species *plantarum*. Miller combined them into *Opuntia ficus-indica* in 1768. In the recorded history of the Old World, *O. ficus-indica* was certainly known at the beginning of the 16th century (*12*), and it is believed that this species accompanied Columbus in his first return to Lisbon in 1493. The cladodes were certainly a novelty to late 15th century Europeans (*23*) and were widely included in ships′ stores as insurance against scurvy (*20*). This practice is thought to have contributed greatly to the present naturalized range of nopal throughout arid and semiarid habitats of the world (*24–26*).

Biology and Botanical Aspects

Biology

Nopal plants are characterized by developing a superficial and fleshy root system with a horizontal distribution, which depends mainly on soil type and culture condition management. In general, the root system of these plants contributes to drought tolerance in three main ways: restraining the root surface, and reducing water permeability absorbing rapidly the low amounts of water provided by light rains and finally by reducing the transpiration thanks to a high negative root potential (*27*).

Cladodes also named as “stems”, “cactus pads”, “cactus vegetable”, “phylloclades”, “nopales” or “pencas”, are the structures that replace leaves with photosynthetic function, that are composed of a white medullar parenchyma (core tissue) and the chlorophyll containing photosynthetically active parenchyma (cortex tissue). The stems are covered with spines (modified leaves) and multicellular hairs and trichomes which form part of what is called “areole”, a structure characteristic of all cacti plants (*28*). Stomata are regularly distributed on both sides of the surface of every cladode and are randomly disposed. *O. ficus-indica* presents between 15 or 35 stomas per mm^2 (*29*). The areoles are oval shaped structures localized 2 mm beneath the skin surface; under proper environmental conditions, from them, the new cladodes, flowers or roots will appear from the meristematic tissue underlying in the areoles. For *O. ficus-indica* these structures are distributed in a helical way, and develop spines instead of leaves (*27*).

Recent anatomical and structural analyses reveal that cladodes of *O. ficus-indica* are typically fleshy with a thick hard skin surrounding an outer layer of chlorenchyma and a central core (*30*). The vascular tissue lies in a single layer at the junction of the green chlorenchyma tissue and the colorless central core

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tissue. The stomata are sunken and the chlorenchyma consists of thin-walled parenchyma containing green plastids and abundant starch. In accordance with previous reports, the cladodes of *O. ficus-indica* contain significant amounts of calcium oxalate crystals and they are present in all nopal tissues. Starch is also usually present in freshly harvested material, and there are always mucilage ideoblasts. The skin material comprises the cuticle, underlying hypodermis, stomatal complexes, and an almost continuous layer of calcium oxalate crystals. Flesh parenchyma derived from the chlorenchyma or central core contains starch associated with calcium oxalate crystals. Variations may reflect agronomic and environmental factors as well as the age of the cladodes used in the studies; younger cladodes have higher carbohydrate content (*31*).

The prickly pear, also named as "tuna" or "fico d′india" is a berry with oval elongated shape weighing 67-216 g. This fruit can be found in many different colors such as white, orange, yellow, red and purple, all of which is due to the presence of betalains (*28*, *32*). The thick pericarp is covered with small-barbed spines hosting a juicy pulp with 150 -300 non-edible seeds. The latter accounts for 3-7%, on a weight basis, followed by the pericarp and mesocarp (36-48%) and the edible pulp (39-64%) (*19*, *32*). Frequently, the fruits of *Opuntia* spp have two types of sterile seeds. The lack of viability in seeds could be related with problems in the ovule or with a young adventitious embryo (*27*). Habibi *et al.* developed some structural studies on the pericarp of *O. ficus-indica* seeds (*33*); also they observed an important amount of lignin (20%) as well as polysaccharides (62%) including cellulose (35%). The structural study of pericarp of *O. ficus-indica* seeds showed that it is mainly made up of spindle shaped sclerenchyma fibers and consists of a natural xylan-cellulose-lignin composite. After lignin elimination, the xylan and cellulose were studied by X-ray and CP/MAS ^{13}C NMR spectroscopy. Water induced a better organization of supramolecular structure of isolated xylan. As components of the seed pericarp, cellulose fibers interact with xylan polymers causing these to adopt a conformation different to the one observed for xylan both in dry or hydrated form.

Similarly to other Opuntia species, *O. ficus-indica* reproduces sexually and propagates vegetatively (*34*). Cladode multiplication is the most extensively used cultivation technique and adventitious roots are developed from the areoles at the portion of the cladode making contact with the soil, allowing rooting as well as water and nutrient absorption. The vegetative bud sprouting starts from the areoles not in contact with the soil and a new individual begins to grow (*35*). Vegetative multiplication appears to be more efficient than sexual reproduction for plant recruitment. Both sexual reproduction and asexual propagation seem to have contributed to the ecological and evolutionary success of the genus, but empirical evidence is lacking (*9*).

Distribution and Production

More than 360 species represent the largest genus of Cactaceae known as Opuntia. The geographic distribution of this plant is large, being found from Peace River in north Canada to the very south of the Argentinean Patagonia; also

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in coastal dunes at sea level and up to 5,100 m in Peru (*36*). This genus includes several species that although originated in North and South America, some are relatively recently distributed throughout the world (*37*). Mexico and Italy are the most important cactus pear producers (*38*). In Mexico about 90% of the marketed fruits are produced from six cultivars, namely Reyna (the most diffused cultivar), Cristalina, Chapeada, Naranjona, Amarilla montesa and Roja pelona. Other cultivars are marginally cultivated (*39*). Cactus pear production in Italy is exclusively based on three cultivars, Bianca (white), Gialla (yellow) and Rossa (red). Other varieties are also cultivated in North Africa, Israel (Ofer), the United States (Andyboy, similar to Italian Rossa) (*38*) and South America (*19*). Other spineless accessions, the Burbank cultivars, are cultivated in South Africa. These genotypes were imported in the early 1990s from the United States (*40*).

According to Inglese *et al.* (*41*) nopal is commercially cultivated for fruit production in southern Italy, particularly in the island of Sicily, where more than 4000 ha of specialized plantations produce 60 000 t of fruits. More than 90% of the Italian production comes from the cultivars Gialla and Rossa. In Mexico, according to the Mexican governmental data, the overall production of nopal cladodes, “nopalitos” used for human consumption has maintained an average of approximately 700 000 t/year, concentrating the production in various states, mainly in Distrito Federal, Estado de Mexico, Morelos, Zacatecas, Tamaulipas, Baja California and Guanajuato (*42*).

Chemical Composition

Cladodes

Guevara-Figueroa *et al.* (*43*) evaluated the proximal composition of some *Opuntia* spp cultivars. The composition of these plants is described in Table I. Wild nopal Blanco had the highest protein content (19% dw). Cristalino and Tapon-II cultivars showed the highest lipid concentrations (1.5 and 1.8%, respectively). Among cladodes, differences were found in carbohydrate content, where the highest value was found in Morado (80.9%) and the lowest value in Tapon-II (42.4%).

Luo *et al.* (*44*) analyzed the petroleum ether extract of *O. ficus-indica* var Milpa Alta by GC/MS. A total of 26 compounds were identified, representing 95.6% of the total extract. Phytosterol (36.03%) was the highest component, and polyunsaturated fatty acids (18.57%) represented the second largest group, followed by phytol (12.11%) and palmitic acid (13.54%) (Table II). The folate content in cladodes of nopal (*O. ficus-indica*) by a microbiological assay, using *Lactobacillus casei* (ATCC 7469) in extracts that were enzymatically treated to release the bound vitamin were also assessed (*45*). In brief, no statistical differences were found between the techniques employed and the folate content was in the range of 5.0 to 5.62 ng/g of fresh tissue (Table II).

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Table I. Proximate composition of different *Opuntia* spp cultivars

			Component (%)					
Sample	***Species***	***Cultivar***	***Protein***	***Lipids***	***Fiber***	***Ash***	***Carbohydrate***	***Reference***
Cladodes	*Opuntia* spp	Blanco	6.7	0.1	15.0	17.3	60.9	(*43*)
		Manso	16.0	0.1	10.8	18.8	54.3	
		Amarillo	15.1	0.6	6.2	14.9	63.2	
		Blanco	19.0	ND	ND	18.3	62.7	
		Cristalino	9.4	1.5	7.7	14.8	66.6	
		Morado	13.9	ND	ND	5.2	80.9	
		Tapon-I	15.1	ND	ND	18.9	66.0	
		Tapon-II	17.4	1.8	20.4	19.5	40.9	
		Tempranillo	13.4	ND	5.5	19.3	61.8	
Seeds	*Opuntia* spp		14.97	11.46	10.14	7.11	56.32	(*59*)
	O. ficus-indica		5.09	5.32	13.28	1.35	74.96	(*63*)

All values are expressed as dry weight basis, ND: not determined.

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Table II. Amino acid, fatty acid, vitamin and mineral content in *Opuntia spp.*

Component	*Cladode [1] (g/100g) fw*	*Fruit juice[1] (mg/L) fw*	*Seed[2] (g/100g protein)*	*Casein[3]*	*FAO/WHO[4]*
Amino acid					
Alanine	0.6	87.2	3.45	3.7	
Arginine	2.4	30.5	4.81		
Asparagine	1.5	41.6			
Asparagine acid	2.1	ND			
Glutamic acid	2.6	66.1	15.73		
Glutamine	17.3	346.2			
Glycine	0.5	11.33	3.67		
Histidine	2.0	45.2	2.26	3.2	
Isoleucine	1.9	31.2	4.50	5.4	
Leucine	1.3	20.6	7.21	9.5	4.8
Lysine	2.5	17.4	4.93	8.5	4.2
Methionine	1.4	55.2	0.51	3.5[a]	2.2
Phenylalanine	1.7	23.3	3.81	11.1[b]	2.8
Serine	3.2	174.5	6.14	ND	ND
Threonine	2.0	13.3	1.11	4.2	4.0
Tyrosine	0.7	12.3	2.24	ND	4.1
Tryptophan	0.5	12.6	ND	1.4	ND

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Component	*Cladode [1] (g/100g) fw*	*Fruit juice[1] (mg/L) fw*	*Seed[2] (g/100g protein)*	*Casein[3]*	*FAO/WHO[4]*
Amino acid					
Valine	3.7	39.4	4.37	6.3	4.2
Cystine	ND	ND	0.27	ND	ND
Fatty acids	***Cladode[5] (%)***	***Seed oil [6] (%)***			
Phytosterol	36.03				
Polyunsaturated fatty acids	18.57				
Phytol	12.11				
Linolenic acid	6.0				
Linoleic acid		61.01			
Oleic acid		25.52			
Palmitic acid	13.54	12.23			
Myristic acid		0.13			

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Table II. (Continued). Amino acid, fatty acid, vitamin and mineral content in *Opuntia spp.*

Fatty acids	***Cladode[5] (%)***	***Seed oil [6] (%)***	
Stearic acid		0.15	
Arachidonic acid		0.95	
Vitamins	***Cladode[1] (fw)***	***Fruit pulp[1] (fw)***	
Vitamin C	7-22 mg/100 g	12-81 mg/100 g	
Carotenoids	11.3-53.5 µg/100 g	0.29-2.37 µg/100 g	
Folates[7]	5.5 ng/g		
Minerals	***Cladode[1] (g/100 g dw)***	***Fruit pulp[1] (mg/100 g fw)***	***Seed[6] (mg/kg flour fw)***
Calcium	5.64	12.8-59	471.2
Magnesium	0.19	16.1-98.4	117.3
Potassium	2.35	90-220	532.7
Phosphorus	0.15	15-32.8	1,627.5
Sodium	0.4	0.6-1.1	71.3
Ferrous	0.14 µg	0.4-1.5	11.7

ND: not determined; [1] References: (*52*); [2] (*59*); [3] (*60*) [4] (*61*); [5] (*44*) [6] (*63*) [7] (*45*); **fw:** fresh weight basis; **dw:** dry weight basis. [a] Methionine + Cysteine; [b] Phenylalanine + Tyrosine.

Bensadon *et al.* (*46*) evaluated the chemical composition of cladode and prickly pear fruit by-products. They compared two Mexican commercial cladode cultivars, Atlixco and Milpa Alta, as well as two prickly pear fruits cultivars, Alfajayucan and Pelón Rojo. According to these authors, some food industry steps generate a large amount of by-products. In general, these by-products are expected to be natural materials rich in dietary fiber, minerals and antioxidant bioactive compounds, thus they could be re-employed in the food industry as a source of functional ingredients. By-products are the outer coating of cladodes and fruits, which are removed before food preparation, and contain spines and a large quantity of glochids and pulp. In this study, the amount of by-products obtained from cladodes and fruit was approximately 17 and 53% (fresh weight, respectively). The total dietary fiber was significantly higher in cladodes than fruits. No significant differences were observed between cultivars (Atlixco and Milpa Alta) with regard to soluble and insoluble dietary fiber (SDF, IDF) in the case of cladodes, whereas in the case of fruits IDF was significantly higher (80%) in Alfajayucan than in Pelón Rojo. Both cladodes and fruits contained high concentration of soluble dietary fiber (SDF).About 15% of the fiber in the two cladode cultivars was soluble whereas samples of Alfayucan and Pelón Rojo fruit cultivars contained 18 and 29% of SDF, respectively. Total carotenoids were found in the order of 21 to 22 mg/g dry matter for both cladode cultivars and between 16 and 15 mg/g dry matter for both prickly pear fruit cultivars. Finally, these authors (*46*) consider that by-products from cladode and fruits of *Opuntia* spp could be utilized as potential functional food ingredients, mainly thanks to their good quality dietary fiber and natural antioxidants.

Contreras-Padilla *et al.* (*47*) evaluated the oxalate and calcium contents in nopal cladodes (*O. redonda*) at different maturity stages. According to this study, calcium content in nopal increases as a function of maturity, ranging from 17.4 mg/g dry weight at 40 days to 34.4 mg/g at 135 days, showing an increase of 97%. The accumulation of calcium in these plants can be related to heat stress, and calcium is able to mitigate heat stress effects by improving stomatal function and other cell processes because this mineral is implicated in many signal transductions that control stomatal aperture (*48*). On the other hand the calcium bioaccessibility in *O. ficus-indica* (L.) Mill cultivars Milpa Alta and Atlixco was assessed by evaluating the effect of cooking, using an *in vitro* digestion and dialysis method (*49*). The studied cladodes had a high content of calcium. However, only 16 and 9% of total calcium was dialyzable in Milpa Alta and Atlixco cultivars, respectively. While the 14% of total calcium in Milpa Alta could be bioavailable, the Atlixco cultivar had only 3% availability. The ionic dialyzable calcium (IDC) has nutritional significance because calcium must be in a soluble form, generally ionized as a prerequisite for its absorption in the upper intestinal tract (*50*). The percentage of IDC was significantly higher in Milpa Alta (90%) than in Atlixco (33%). However, the non-dialyzed calcium (NDC), constituted the major fraction of calcium in the two cultivars (84% - 91% of total calcium). Cooking conditions applied did not cause significant changes in total oxalate or in the bioaccessibility in any of the cultivars studied. Also in this study it was observed that ash content contributed to 18% of dry matter, K being the most abundant mineral in both cultivars, followed by Ca and Mg. Other minerals

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(Na, Cu, Fe, Mn, Zn) were also present but the content was low. We should note that the cladodes are a good source of calcium (1701-1966 mg/100 g dry matter), in comparison with other vegetables (*49*).

Also, cladode sugar composition was analyzed (*30*). In whole dry cladodes there was a prevalence of glucose and galacturonic acid, while arabinose, galactose, mannose, xylose and rhamnose were present in smaller quantities (Table III). A high level of glucose in the cladodes, which was estimated to be 42%, was detected. Samples treated with I_2/KI were stained blue/brown, suggesting that significant amounts of either starch or xyloglucan were present; they had a starch content of values vary from 5.0 to 6.6. The soluble solid content of 12 to 17% is greater than that present in other fruits such as prunes, apricots and peaches (*52*). They are considered as good sources of vitamins, amino acids and betalains. In this sense, vitamin E, tocopherols and *β*-carotene are found in the lipid fraction of these fruits (seeds and pulp) at 89.5 ± 4.4 mg/g. It was interesting to note a high percentage of calcium oxalate and Klason lignin (16%) in the cladodes.

Table III. Monosaccharide composition of cladodes from *O. ficus-indica* [a]*. (Data from Reference (*30*). Copyright 2009 ACS)

	Whole dry cladodes-monosaccharides
Rhamnose	7.13
Fucose	0.74
Arabinose	39.64
Xylose	18.64
Mannose	13.64
Galactose	33.69
Glucose	153.15
Gal A	96.26
Total (µg/mg)	362.89

[a] Composition is expressed as total µg/mg of dried material; Gal A: galacturonic acid.

Recently, the chemical composition of nopal powders from three different maturity stages (50, 100 and 150 days), which were obtained from three different drying processes (freeze dried, forced air oven and tunnel), were evaluated (*51*). Accordingly, the highest content of protein, soluble fiber and fat were retained in nopal powders by the freeze dried process. In brief, the insoluble fiber content, chroma and lightness were not affected with any of the three drying processes. Fiber content, chroma and lightness were not affected with any of the three drying processes. Finally, soluble fiber content showed a decreasing trend with relation to nopal age; in the case of insoluble fibers and ash content, the effect was the contrary.

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Fruits

Cactus pear fruits exhibit ascorbic acid contents in the range of 20 to 40 mg/100 g fresh weight and titratable acidity of 0.03 to 0.12%. Also prickly pear fruits are good sources of calcium, potassium and magnesium. The total caloric value of this fruit is 50 kcal/100 g, which can be compared to pears, apricots and oranges. Amino acid, fatty acids, vitamin and mineral contents of nopal fruits are described in Table II.

Cactus pear pulp also contains water soluble betalain pigments that are accumulated into the vacuole. Betalainic phytochemicals are nitrogen-containing pigments occurring in the Caryophyllales (*28*). Pigment analyses have indicated that the main betacyanin and betaxanthin in cactus pear are betanin and indicaxanthin. Modifications in the proportion of these pigments may be reflected in pulp color (*53*). In this sense, Felker *et al.* (*54*) evaluated the color inheritance pattern for cactus pear (*O. ficus-indica*) fruits. In this work, degenerated primers were used to obtain partial genomic sequences of two major genes in the biosynthetic pathway for betalains. Differences in the genomic DNA between colored versus non-colored cultivars were not found. According to these authors, regulatory mechanisms seem to independently control pigmentation of *O. ficus-indica* fruit tissues for inner core, peel and epidermis. Core pigmentation occurs first and well before fruit maturity and peel pigmentation. Peel pigmentation is fully developed at maturity, presumably related to maximum soluble solids. Epidermal pigmentation appears to be independent of core and peel pigmentation, perhaps because of light stimulation. Similar control mechanisms exist through transcription factors for the major enzyme regulating anthocyanin production in grapes (*54*). Also, other mechanisms such as fruit ripening control color appearance in these as well as in other fruits. They are the direct reflect of many gene expression changes, which can be either positive or negative, and constitute the evidence that the expression of at least some of them is confined to ripening fruit tissues. In brief, the problems of ripening encompass the questions of metabolic control, communication between organelles, mechanism of action of plant hormones and developmental regulation of gene expression (*55*). The wide range of environmental conditions in which fruits grow and ripen, results in a great variability in fruit yield and fruit ripening time, fruit quality (shape, size, flesh percent, sugar content, flavor and taste). Fruit size depends on crop yield per plant and per cladode (*56*) as well as on crop management in terms of irrigation and fruit thinning (*57*), while other factors such as plant architecture, fruiting cladode position within the canopy and cladode characteristics have been poorly studied as sources of variability in fruit yield and quality (*58*). The effect of environmental conditions on fruit quality of *O. ficus-indica* cultivars Rossa and Gialla, has been analyzed (*41*). In general, fruit weight, shape and total soluble solid content significantly changed with the environmental conditions (site, altitude) while flesh percent, pH and total titratable acidity did not. Cultivars had a significant influence only on fruit weight and seed content. Fruit weight changed greatly within the tree, while total soluble solid content and flesh percent variability was much reduced. Fruit weight decreased with fruit number per tree and both fruit weight and total soluble solid content decreased with more than six fruits per cladode. Light interception and cladode dry weight were the main sources of fruit dry weight variability and sugar content, while cladode surface area was poorly related to fruit quality. The role of cultivars in determining fruit quality did not change with site and, moreover, the sensory analysis was unable to discriminate for cultivar and environment.

Seeds

Nassar (*59*) evaluated the chemical composition of *O. ficus-indica* seeds focusing on protein content and nutritional value. The prickly pear seed (PPS) presented high contents of protein, lipids, fiber, ash and carbohydrates (Table I). Several studies have revealed that prickly pear seeds are considered an untraditional source of protein. However, all essential amino acids levels of PPS flour and protein concentrate were above than those reported for the FAO/WHO reference protein (*60*, *61*), except for methionine, threonine and tyrosine (Table II). Protein solubility is one of the most important functional properties because it influences other functional properties. In this sense, evaluations of protein solubility done at pH 4 and 5 demonstrated a sharp decrease for both PPS and protein concentrate, although there was a good solubility in acid and alkaline pH regions, which is important for food formulations. Moreover, at highly acidic and alkaline pH´s, the protein acquires net positive and negative charges, respectively, favoring the repulsion of the molecules and thereby increasing protein solubility (*62*). Also, emulsion capacity results showed that this property was pH dependent and that alkaline pH improved the emulsion capacity more than acid pH.

The nutritive value and chemical composition of prickly pear seeds (*O. ficus-indica*) growing in Turkey was evaluated (*63*). According to them, the proximate composition of Opuntia seeds was: crude protein 5.09%, crude lipids 5.32% and fiber 13.28% (Table I). Opuntia seeds also contained significant amounts of important minerals. Mineral composition was calcium 471.2, magnesium 117.3, potassium 532.7, phosphorus 1,627.5, sodium 71.3 and iron 11.7 mg/kg dw (Table II). Oil content found in the evaluated nopal seeds differed with those reported previously (*59*, *64*). The peroxide value of the Opuntia seed oil was 1.63 meq O_2/kg, the saponification value was 181.3, lower than those of sunflower and corn oil (*65*), and slightly higher than those of Opuntia seed oil (*66*). However, results differed with literature values (*66*, *67*); differences in oil content and saponification values may be due to growth conditions, varieties, genetic factors, harvesting times, soil properties, or geographical variations in nopal plants. According to this study (*63*) the *O. ficus-indica* seed oil contains linoleic acid as the major fatty acid (61.01%), followed by oleic (25.52%) and palmitic acids (12.23%). Also, myristic, stearic and arachidonic acids were detected in *O. ficus-indica* seed oil in low amounts (Table II). On the other hand, other authors have shown that this seed oil contains 67.20% linoleic, 18.0% oleic, 10.40% palmitic and 3.0% stearic acids (*68*). The observed differences in the concentration of linoleic, oleic and palmitic acids could be due to the fruit maturity degree, which is in accordance to the literature, where it has been stated that there is an increase in saturated fatty acid content towards the end of fruit

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maturation (*64*). The fatty acid composition of prickly pear oil was close to those of sunflower and grape seed oils.

Flowers

The chemical composition of flowers from *O. ficus-indica* has also been analyzed. De Leo *et al.* (*69*) analyzed the purified methanol extract of *O. ficus-indica* flowers by means of HPLC-PDA-ESI-MS/MS. The results revealed the presence of secondary metabolites belonging to the flavonol glycoside class, as indicated by absorbances at 255-265 and 350-355 nm. According to the MS/MS spectra of all precursor ions, the presence of three different aglycone skeletons, corresponding to kaempferol, quercetin and isorhamnetin respectively was detected. All compounds were glycosides, carrying one, two or three sugar units and were identified as follows: quercetin-3-O-rutinoside, kaempferol 3-O-rutinoside, quercetin 3-O-glucoside, isorhamnetin 3-O-robinobioside, isorhamnetin 3-O-galactoside, isorhamnetin 3-O-glucoside and kaempferol 3-O-arabinoside. The total amount of flavonoids of *O. ficus-indica* flowers was 81.75 mg/ g of fresh plant material. Isorhamnetin 3-O-robinobioside was the major component (52.22%), and it was followed by isorhamnetin 3-O-galactoside (11.98%), isorhamnetin 3-O-glucoside (8.86%), quercetin 3-O-rutinoside (8.67%), quercetin 3-O-glucoside (5.47%), kaempferol 3-O-rutinoside (4.89%), kaempferol 3-O-arabinoside (3.96%), and finally two unidentified compounds. Also 18 volatile compounds were identified which represent 84.2% of the whole volatile profile in nopal flowers. The main constituents were found to be germacrene D (12.6%), followed by 1-hexanol (12.3%), n-tetradecane (9.1%), and decanal (8.2%). Monoterpene hydrocarbons were completely absent, while oxygenated monoterpenes represented 16.5%; on the contrary sesquiterpene hydrocarbons occurred at 18.0%, with germacrene D as the major component, while there was no presence of oxygenated sesquiterpenes. Open chain aldehydes with non-terpenic structure (10.7%) were represented by nonanal (2.5%) and decanal (8.2%), respectively. Open chain hydrocarbons with non-terpenic structure containing 13, 14, 15 and 16 carbon atoms represented 20.6% of the total constituents. Three open chain non-terpenic alcohols occurred at 17.2%. 2-Ethyl hexyl acetate was at 1.2%. This is the first report on flower volatile composition of *O. ficus-indica*.

Nutraceutical Compounds and Their Pharmacological/Medical Applications

Antioxidants

Phenolic compounds can be defined as substances possessing an aromatic ring, carrying one or more hydroxyl groups including their functional derivatives (*70*). Plants contain a large variety of phenolic derivatives including simple phenols such as phenylpropanoids, benzoic acid derivatives, flavonoids, stilbenes, tannins, lignans and lignins. These compounds constitute about one third of the dietary phenols and act as antibiotics, natural pesticides protective agents against

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ultraviolet light and insulation materials to give plants stability. Therefore, many beneficial health effects exerted by plant foods are associated with the presence, type and content of phenolic compounds (*71*). Color in prickly pears from Opuntia is due to the presence of betalains, which are N-heterocyclic water-soluble pigments deposited in vacuoles. Betalamic acid is the common precursor of these compounds which consists of a 1,7-diazaheptamethin system responsible for a canary yellow color. The condensation of betalamic acid with cyclo-Dopa can yield betanidin, a common aglycon precursor of red betacyanins. Betanidin in turn may be glycosylated and/or acylated yielding 29 structures (*72*). The most important cactus betalains are betacyanins and betaxanthins. Betacyanins display two absorption maxima, one in the UV-range between 270 and 280 nm and a second one at about 535-538 nm depending on the solvent system (*73*). Betalains are pigments with high antioxidant properties; it has been stated that natural food colors can inhibit cell proliferation in a variety of human tumor cells (*74*, *75*) (Figure 1).

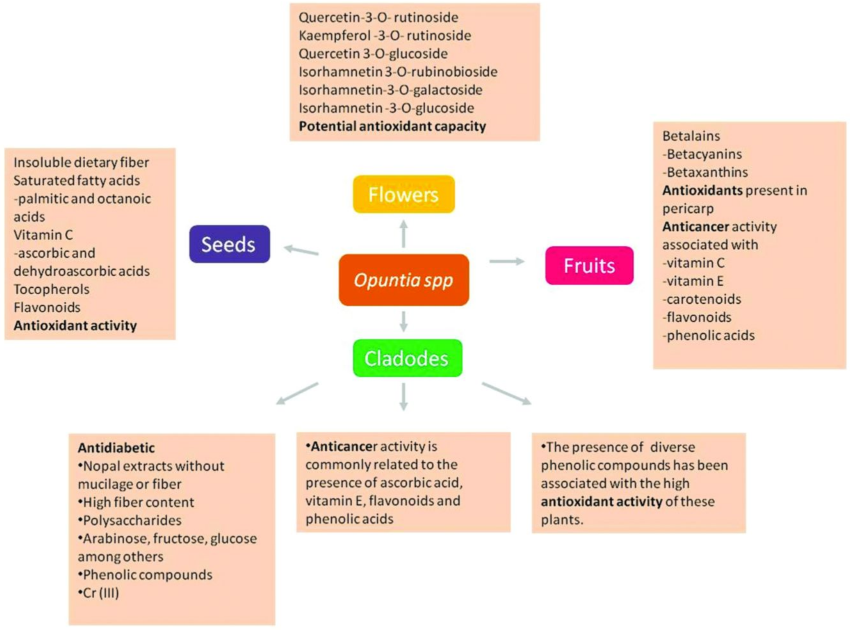
Quercetin-3-O- rutinoside
Kaempferol -3-O- rutinoside
Quercetin 3-O-glucoside
Isorhamnetin 3-O-rubinobioside
Isorhamnetin-3-O-galactoside
Isorhamnetin -3-O-glucoside
Potential antioxidant capacity
Insoluble dietary fiber
Saturated fatty acids
-palmitic and octanoic acids
Vitamin C
-ascorbic and dehydroascorbic acids
Tocopherols
Flavonoids
Antioxidant activity
Flowers
Seeds
Opuntia spp
Fruits
Cladodes
Betalains
-Betacyanins
-Betaxanthins
Antioxidants present in pericarp
Anticancer activity associated with
-vitamin C
-vitamin E
-carotenoids
-flavonoids
-phenolic acids
Antidiabetic
•Nopal extracts without mucilage or fiber
•High fiber content
•Polysaccharides
•Arabinose, fructose, glucose among others
•Phenolic compounds
•Cr (III)
•**Anticancer** activity is commonly related to the presence of ascorbic acid, vitamin E, flavonoids and phenolic acids
•The presence of diverse phenolic compounds has been associated with the high **antioxidant activity** of these plants.

Figure 1. Nutraceutical activities described in the Opuntia genus.

Morales *et al.* (*76*) evaluated nutritional and antioxidant properties of pulp and seeds of two xoconostle cultivars (*O. joconostle* F.A.C. Weber ex Diguet and *O. matudae* Scheinvar) of high consumption in Mexico. Xoconostle fruit pulp contained a considerable amount of digestible carbohydrates, while the seeds had a significant predominance of non digestible compounds (insoluble dietary

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fiber), protein, lipids and ash contents. Lipid levels in xoconostle seeds of *O. joconostle* and *O. matudae,* respectively, were 2.45 and 3.52 g/100 g fw. At least 19 fatty acids were identified and quantified in the seeds of both cultivars, while in the pulp only 16 fatty acids were characterized. Two saturated fatty acids, palmitic acid and octanoic acid, were found in significant amounts. Two active vitamin C forms were identified: ascorbic acid and dehydroascorbic acid. Total tocopherols content was higher in seeds of both cultivars than in the pulp. Four isoforms (α, β, γ and δ tocopherols) were identified; seeds presented high amounts of γ-tocopherol whereas α-tocopherol was the main isoform in the pulp with relatively low values. Xoconostle seed presented the highest phenolics and flavonoid contents particularly *O. matudae* (phenolics 59.48 mg gallic acid equivalents (GAE)/g of extract and flavonoids 58.40 mg catechin equivalents (CE)/g of extract). The total polyphenol content in these cultivars was three fold higher than the one reported previously in other cultivars. Phenolic compounds help to protect plants against UV light and act as defenses against pathogenic microorganisms in plants. This type of protection might be used by these fruits that remain attached to mother plants for several months without deteriorating, which in turn might explain the elevated contents of phenolics and flavonoids when compared to other Opuntia fruits. The highest antioxidant properties of seeds are in agreement with their highest phenolics and flavonoids contents. Seeds of *O. joconostle* presented the highest diphenylpicrylhydrazyl (DPPH) scavenging activity and reducing power activities, whereas pulp of both varieties presented the lowest phenolics content and the worst antioxidant activity.

The phenolics, betacyanins and antioxidant activity in *O. joconostle* fruits was evaluated (*72*). The total phenolic content found was 2.07 mg GAE/ g of fresh pericarp. Also the flavonoid content was assessed and the results showed that fresh pericarp presented the largest amount of flavonoids, followed by endocarp and mesocarp. This may be due to the action of light and other environmental factors that can induce flavonoid synthesis in the plant. Interestingly, the highest betacyanin content was found in endocarp followed by mesocarp and pericarp. The whole fruit concentration was 7.57 mg betanin/100 g fw.

The antioxidant compound contents in three different cactus pear fruit cultivars grown in Spain, *O. ficus-indica*, *O. undulata* and *O. stricta,* were analyzed (*77*). All the *Opuntia* spp. tested showed significant amounts of flavonoids, quercetin being the predominant type followed by isorhamnetin, luteolin and kaempferol. On the other hand, the highest total flavonoid content was found in *O. stricta* fruits, while *O. undulata* fruits showed the lowest value. Quercetin derivates accounted for 54.3-58.7% of total flavonoids, isorhamnetin derivatives for 18.8-31.7%, luteolin derivatives for 5.4-11.5% and kaempferol derivatives for 4.8 – 11.0% of total flavonoids. Considering betaxanthin levels, only in *O. ficus-indica* (25.4 mg/100 g fresh fruit) and in *O. undulata* (17.8 mg/100 g fresh fruit), were observed, being indicaxanthin the main betaxanthin identified. In general, all three cactus pear species analyzed contained taurine (7.7-11.2 mg/100 g fresh fruit); this sulfur containing amino acid functions as a neuroinhibitory transmitter and is considered a cell protective compound (*77*).

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Antidiabetics and Obesity

Diabetes mellitus is a metabolic disorder of multiple etiologies in which chronic hyperglycemia is caused by defects in either secretion or action of insulin or alterations in both of these processes. As a result, there are disturbances in the carbohydrate, fat, and protein metabolism. Also resistance and a relative deficiency of insulin or impaired insulin secretion that can be followed by insulin resistance can lead to the onset of type 2 diabetes (*78*, *79*). Many plants around the world are used for their beneficial effects *versus* this and other diseases. Namely in Mexico, traditional and ancestral medicine favors the utilization of different cultivars of nopal (*Opuntia* spp) from generation to generation in type 2 diabetes and other diseases. Therefore, many scientific reports have emerged in recent years concerning the potential use of Opuntia cladodes and fruits, in the type 2 diabetes treatment.

Luo *et al.* (*44*) evaluated the chemical composition and antidiabetic activity of *O. ficus-indica* cultivar Milpa Alta extracts. In these extracts a total of 26 compounds were identified and were described in a previous section of this chapter (Table II). Nevertheless, interestingly three new compounds were observed; monobutyl 2-(4-hydroxybenzyl) tartrate, ethyl 2-(4-hydroxybenzyl) tartrate and diethyl 2-hydroxy-2-(4-hydroxybenzyl) succinate. However, the potential activity of these compounds was not described in this report. The antidiabetic activity of the extracts was evaluated in streptozotocin (STZ) induced diabetic mice. The level of blood glucose in the Opuntia treated groups was significantly lower than the control group. Body weight increased regularly versus a slight decrease in mice from the control group. In general the authors suggest that these plant extracts could serve as good adjuvant in the present armamentarium of antidiabetic drugs (Table IV).

Yoon *et al.* (*80*) analyzed the effect of a nopal (*O. ficus-indica*) complex in the blood glucose metabolism in *db/db* mice. The complex was composed by 65% nopal and other Asian medicinal plants. In general, the nopal complex significantly reduced the food intake and increased total water intake. Also, fasting blood glucose levels decreased significantly after 2 weeks of feeding. In addition after 4 weeks of nopal feeding, pancreatic islet integrity of *db/db* mice was improved, and pancreatic cell proliferation increased significantly; this took place mainly in β cells of the pancreatic islets followed by insulin secretion induction, elevating plasma insulin levels and glucose tolerance. The authors suggest that a compound present in nopal might have sulfonylurea like activity, but on the other hand, this complex elevated plasma insulin levels not by improving pre-synthesized insulin secretion, but by recovering overall β-cell function accompanied by islet cell proliferation. In this sense, these authors assumed that the hypoglycemic effects of this complex may operate differently from conventional diabetic drugs such as sulfonylureas (Table IV)

The antidiabetic effect of polysaccharides from *O. dillenii* was assessed (*81*). Other studies on these compounds derived from *Opuntia* spp contain arabinose, xylose, fructose, glucose, galacturonic acid, and rhamnose units and exhibit various functional properties such as protective effects against H_2O_2 induced damage, free radical-scavenging and anti-inflammatory activity,

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antitumor activity, blood lipid-lowering effects, and wound-healing activity (*81*, *82*). Therefore, these authors reported the antidiabetic effect of *O. dillenii* polysaccharide (ODP)-la in mice with STZ induced diabetes.

Three kinds of ODPs were isolated and administered to the mice during 3 weeks. ODP-la administration during this time significantly decreased the intake of food and water (Table IV). It also decreased the fasting levels of blood glucose, total cholesterol, triglycerides, plasma urea nitrogen and malondialdehyde. Interestingly, it significantly increased mice body weights, hepatic glycogen levels, high density lipoprotein cholesterol levels and hepatic superoxide dismutase and glutathione peroxidase activity in diabetic mice; all of which are positive biomarkers of this disease. Accordingly, these authors suggest that ODP-la exerts its antihyperglicemic effect by protecting the liver from peroxidation damage and maintaining tissue function, thus improving sensitivity and response of target cells to insulin

The antihyperglycemic effect of *O. streptacantha* Lem in STZ diabetic rats was also studied (*2*). The experiments were done with two extract samples: the first one was prepared in the traditional way producing a liquefied extract and the second one was a filtrate of the first extract containing a newly isolated compound (4-hydroxy)-phenyl acetic acid. This study confirms that the anti-hyperglycemic effect of nopal is independent of the fiber or mucilage content, demonstrated by testing the filtrated extract which lacks fibers and pectins; it has been commonly suggested that the anti-diabetic effect of Opuntia may be a result of the high fiber and pectin content which may decrease carbohydrate absorption (Table IV.).

Hahm *et al.* (*83*) reported that *O. humifusa* stems can lower blood glucose and cholesterol levels in STZ induced diabetic mice. The experiments were developed in STZ induced diabetic mice fed with growing concentrations of nopal. In general the administration of *O. humifusa* had no effect on daily water intake, food intake or food efficiency (Table IV.) However, nopal treated animals presented significantly lower fasting glucose levels as well as lower serum total cholesterol and low density lipoprotein (LDL) cholesterol levels. Animals treated with nopal (250 and 500 mg/kg body weight) presented higher high density lipoprotein (HDL) cholesterol levels, also lower serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations were observed. In addition, treated animals showed a significant increase in relative β cell volume. These authors suggest that dietary fiber content and possibly other carbohydrate components in *O. humifusa* may contribute to the glucose lowering effect observed in a similar way of other high-fiber containing foods (*83*) (Table IV).

It has been reported that a generous intake of dietary fiber reduces the risk for developing obesity and aids in weight loss. Cross-sectional and prospective cohort studies in men and women indicate that high-level fiber consumption reduces risk for gaining weight or developing obesity. Conversely, obesity is associated with diabetes, hypertension, osteoarthritis, and heart disease. The obesity incidence has increased at an alarming rate in recent years, becoming a worldwide health problem, with incalculable social costs (*46*, *51*, *83*).

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Table IV. Nutraceutical activities of different compounds or extracts found in the Opuntia genus

	Nopal cultivar	*Extract/ Compound*	*Observations*	*Reference*
Antidiabetic	*O. ficus-indica* Milpa Alta	Extracts with 26 compounds	Antidiabetic effect was observed in STZ induced diabetic rats.	(*44*)
	O. ficus-indica	Nopal complex with other Asian medicinal plants	The antidiabetic effect was attributed to the presence of an specific compound which may operate differently from sulfonylureas.	(*88*)
	O. dillenii	ODP-la ODP-Ib and ODP-II Polysaccharide fractions	ODP-la decreased food intake, fasting blood glucose levels, total cholesterol, triglycerides, plasma urea, nitrogen and malondialdehyde.	(*81*)
	O. streptacantha Lem	Liquefied extract and a filtered extract of nopal cladodes	The positive results over diabetic induced mice were attributed for the first time not to high fiber content but to a new compound present in these extracts.	(*2*)
	O. humifusa	Nopal cladode powders	The overall positive effect was attributed to phenolic compounds and fibers.	(*83*)
	O. ficus-indica *O. dilleni*	Nopal cladodes and fruits	The antidiabetic effect was attributed to high Cr (III) content.	(*84*)

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	Nopal cultivar	***Extract/ Compound***	***Observations***	***Reference***
Anticancer				
	O. ficus-indica	Nopal cladode extract	Favorable restoration response vs micotoxin and a significant prevention against zearalenone genotoxicity evaluated in zearalenone Balb/c mice.	(*4*)
	O. humifusa	Hexane soluble extracts of fruits, cladodes and roots	The use of the extracts over U87MG human glioblastoma cells decreased cellular proliferation.	(*91*)
	Opuntia spp (9 varieties)	Fruit juices	Four mammalian cancer cell lines: Mammary (MCF-7), prostate (PC3), colon (Caco2), hepatic (Hep G2). Moradillo variety showed the best effect over prostate and colon cancer viability without affecting mammary and hepatic cancer cells.	(*92*)
	O. ficus-indica	Cladode extract	A positive effect was observed when utilizing the cladode extract over Balb/c mice treated with aflatoxin B1, reducing the early markers of oxidative stress.	(*93*)

The chromium (III) in cactus pads and its possible role in antihyperglycemic activity has been investigated as well (*84*). Cr (III) plays an important role in glucose metabolism since this trace element forms part of a glucose tolerance factor; a molecule that decreases the resistance of the tissues to insulin action. Glycemic curves revealed that the consumption of nopal cladode and fruit pulp extracts did not produce a significant effect on blood glucose. Nopal cladode extract and fruit pulp administration along with a Cr (III) solution to diabetic mice produced a significant decrease on fasting glucose levels (Table IV). However, evidence from this research does not seem convincing, since it has been described previously in this chapter that plant microelement levels depend in many ways of the geographical area where the plants are cultivated, and of the genetic variety and environmental conditions (namely, soil conditions).

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Anticancer Activity

Natural occurring phytochemicals such as phenolic compounds have been proved to prevent cancer metastasis. Flavonoids are a very large subclass of phenolic compounds that are abundant in food and nutraceuticals. Increasing scientific evidence gathered around the world demonstrates that flavonoids are potent cancer invasion and metastasis inhibitors. Catechin derivatives, (-)-epigallocatechin-3-gallate, (-)-epigallocatechin, (-)-epicatechin-3-gallate and (-)-epicatechin are the most studied compounds in this topic; genistein/genistin, silibinin, quercetin and anthocyanin have also been widely investigated for their inhibitory activities in invasion and metastasis. Some others include luteolin, apigenin, myricetin, tangeretin, kaempferol, glycitein, licoricidin, daidzein and naringenin which are present in vegetable foods and exhibit anti-invasive and anti-metastatic activities. Bioactive compounds are necessary to prevent tumor cells from metastasizing (*85*). Chemoprevention has emerged as an alternative to prevent cancer formation or its progression by means of utilizing natural or synthetic substances and was described as early as 1960s. This concept has been expanded to target all cancer developmental stages; including from prevention of cancer initiation through DNA repair, detoxification, free-radical scavenging and carcinogen metabolism, prevention of tumor promotion and progression through inhibition of proliferation and angiogenesis, induction of apoptosis and differentiation and reduction of inflammation and increase immunity (*86*, *87*). In this sense, scientific evidence concerning the use of nopal as an anticancer agent is emerging.

Zorgui *et al.* (*4*) evaluated the antigenotoxic activities of cactus (*O. ficus-indica*) cladodes against the mycotoxin zearalenone in Balb/c mice. Mycotoxins are natural occurring food contaminants. They cause severe health problems such as endometrial adenocarcinomas and hyperplasia (*88*), as well as severe liver lesions with subsequent development of hepatocarcinoma (*89*, *90*). Nopal cladodes were fed to Balb/c mice affected by mycotoxin exposition. Treatment with nopal cladodes restored favorably the number of polychromatic erythrocytes (citotoxicity evaluation) at a very low dose (25 mg/kg). At increasing nopal concentrations, a significant prevention *versus* zearalenone genotocixity was observed. DNA fragmentation test in treated animal cells revealed that after cladode administration a significant restoration of DNA in a dose dependent manner. The authors attributed this effect to the presence of multiple antioxidant compounds.

Hahm *et al.* (*91*) evaluated the effect of *O. humifusa* extracts over U87MG human glioblastoma cells. Glioblastoma is one of the most common types of malignant tumors in the neurological system. Therefore, hexane and water extracts were obtained from different plant parts: fruits lacking seeds, seeds, roots and cladodes. Cytotoxicity assay results revealed that the water partitioned fraction of *O. humifusa* significantly suppressed cell proliferation in a dose dependent manner. Also, treatment with hexane-soluble extracts (500 µg/mL) of fruit, cladodes and roots decreased U87MG cells proliferation by 49, 55 and 52%, respectively. Also, the effect of these extracts on cell cycle of glioblastoma cells was evaluated. Interestingly, the increase in the number of cells in G1 phase was accompanied by a concomitant decrease in the number of cells in S and G2/M phases. These data suggest that water partitioned fraction of *O. humifusa* might have the potential to arrest U87MG cells in the G1 phase. In addition, the effect of nopal over apoptotic and non apoptotic U87MG cells when treated with the different extracts was evaluated. In brief no significant difference was observed in the number of apoptotic cells, however a 2% increase was found for non apoptotic cells (Table IV).

Also, Chávez-Santoscoy *et al.* (*92*) assessed the phenolic composition, antioxidant capacity and *in vitro* cancer cell cytotoxicity of nine prickly pear (*Opuntia* spp.) juices. Four different mammalian cell lines were used for the *in vitro* analyses, namely mammary (MCF-7), prostate (PC3), colon (Caco2) and hepatic (HepG2). Normal fibroblast (NIH 3 T3) was used as control. Among the four cancer cells tested, the viability of prostate and colon cancer cells were the most affected especially by the Moradillo opuntia fruit juice (Table IV). Nevertheless, this cultivar juice also diminished the growth of normal fibroblasts used as control. The Rastrero cultivar as well, diminished the prostate cancer cells proliferation but not the normal fibroblast viability. In fact, this juice was effective against all four cancer cell lines evaluated; this could be due to the high antioxidant capacity detected which was comparable to pomegranate juice. The Gavia juice was the most effective versus colon cancer cells and to some extent also affected prostate and hepatic cancer cell growth. However, this same cultivar did not show any activity over hormone dependent mammary and fibroblast cells. Juice from Cardon affected prostate and colon cancer cells viability, almost in the same way that Rastrero did. The juices obtained from *O. robusta* or *O. rastrera* were the only ones that diminished the viability of hepatic cancer cells. However according to these authors, further studies are needed to determine which are the anticancer compounds with higher activity and if the *in vitro* results correlate with animal studies.

The chemopreventive effect of cactus *O. ficus-indica* on oxidative stress and genotoxicity of aflatoxin B1 was also evaluated (*93*). Primary liver cancer, also known as hepatocellular carcinoma (HCC) is caused mainly by underlying infections caused by the hepatitis B and C viruses. Aflatoxins are produced as secondary metabolites by *Aspergillus flavus* and *A. parasiticus* fungi. Aflatoxins B1 (AFB1) are the most potent of these toxins which have hepatotoxic and

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hepatocarcinogenic properties. The effect of a nopal cladode extract of 50 mg/kg body weight was evaluated in Balb/c mice by monitoring its effects on oxidative stress, genotoxicity and cell death pathway induced by sub-chronic treatment by aflatoxin B1. Accordingly, the exposure to AFB1 induced a marked increase in malondialdehyde level in the liver. The pre and post-administration of nopal cladode extract along with AFB1 significantly reduced this effect. Also, this treatment reduced significantly the protein carbonyls generation in liver. Finally early markers of oxidative stress were monitored. Therefore, the treatment with cladode extract, after administration of AFB1, reduced significantly the levels of Hsp 70 and Hsp 27, demonstrating the capability of nopal cladode extracts to prevent and protect against oxidative damage, which is likely due to the high content of antioxidant compounds present in these plants.

Other Nutraceutical or Medical Effects

Kim *et al.* (*94*) examined the effect of using a methanol extract of *O. ficus-indica* on neuronal injury. In brief, these authors observed that this extract had a neuroprotective action against neuronal injury induced by *N*-methyl-D-aspartate (NMDA), kainate (KA), and oxygen-glucose deprivation (OGD) in cultured mouse cortical cells. The treatment of neuronal cultures with the methanolic extract inhibited the NMDA; KA and OGD induced neurotoxicity in a dose dependent manner. The butanol fraction of *O. ficus-indica* (300 µg/mL) significantly reduced NMDA, and induced delayed neurotoxicity by 27%. Gerbils were treated with methanolic extracts every 24h for 3 days or during 4 weeks and ischemic injury was induced after the last dose. Neuronal cell damage in the hippocampal CA1 region was evaluated quantitatively at 5 days. When gerbils were given doses of 4.0 g/kg (3 days) and 1.0 g/kg (4 weeks) the neuronal damage in the hippocampal region was reduced by 32 and 36%, respectively. These results suggest that the preventive administration of *O. ficus-indica* extracts may be helpful in alleviating the excitotoxic neuronal damage induced by global ischemia.

Also, Lim (*95*) evaluated the inhibitory effect of a glycoprotein isolated from *O. ficus-indica* cultivar Saboten Makino on activities of allergy-mediators in compound 48/80 which stimulated mast cells. The present study was performed to investigate the anti-allergy potentials of a nopal isolated glycoprotein (90 KDa) on ICR mice (*in vivo*) and on RBL-2H3 cells (*in vitro*). Allergy related factor activities were analyzed: histamine and β-hexosaminidase release; lactate dehydrogenase (LDH) and interleukin 4 (IL4) in compound 48/80 treated ICR mice (*in vivo* analysis). Thus, the authors speculate that this glycoprotein is a natural compound that blocks anti-allergic signal transduction pathway.

Kim *et al.* (*96*) assessed the effect of using an n-butanolic extract of *O. ficus-indica* cultivar Saboten Makino on long term memory in mice. After oral administration of this butanolic extract during 7 days, the latency time in the passive avoidance task was significantly increased relative to vehicle-treated controls. Western blot analysis revealed that the expression levels of brain-derived neurotrophic factor (BNDF), phosphorylated cAMP response element binding protein (pCREB) and phosphorylated extracellular signal-regulated kinase (pERK) were significantly increased in hippocampal tissue after 7 days of butanolic extract administration. Doublecortin and 5-bromo-2-deoxyuridine immunostaining also revealed that this butanolic extract significantly enhanced the survival of immature neurons, but did not affect neuronal cell proliferation in the subgranular zone of the hippocampal dentate gyrus. These results suggest that the subchronic administration of this butanolic extract enhances long-term memory and that this effect is partially mediated by ERK-CREB-BNDF signaling and the survival of immature neurons.

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Applied Molecular Biology and Biochemistry in Opuntia

Available Nucleic Acids Extraction Protocols

DNA

DNA extraction from plant tissues, unlike DNA isolation from mammalian tissues, remains difficult due to the presence of a rigid cell wall surrounding plant cells. Currently used methods inevitably require a laborious mechanical grinding step, necessary to disrupt the cell wall for the DNA release. The field of plant molecular biology is therefore at a disadvantage, especially when an automated high-throughput system for the isolation of PCR ready genomic DNA is required in population genetics, species identification, biodiversity investigation, selection screening, food control and plant biotechnology (*97*). DNA isolation from cacti is notoriously difficult because they contain high amounts of polysaccharides and secondary metabolites which form insoluble complexes with nucleic acids during extraction (*98*). Like in other groups of plants, the secondary metabolites and polysaccharides in cacti inhibit enzyme action (*99*). The polysaccharides are visually evident by their viscous, glue-like texture and they make the DNA unmanageable when pipeting and hard to amplify by the polymerase chain reaction (PCR) (*99*). Therefore the need for an efficient method towards the application of a molecular approach to any species is to develop techniques to extract high quality DNA (*100*).

In this sense, in the late 90´s decade De la Cruz *et al.* (*101*) developed a DNA isolation protocol for 85 different cacti species of 39 genera (subfamilies Pereskioideae, Opuntioideae and Cactoideae). According to this protocol, only a few grams of tissue are required for the extraction and it does not require whole plant destruction to produce high molecular weight genomic DNA (Table V). Hence, the DNA obtained can be amplified consistently by PCR and used for RAPD (random amplified polymorphic DNA) analysis. This method is based in the utilization of the CTAB (cetyl trimethyl ammonium bromide) buffer with some modifications. Fresh tissue from cacti presents large amount of polyphenolics and polysaccharides that co-precipitate with DNA and affect subsequent PCR amplification (*102*). The addition of an initial CTAB extraction step significantly reduced the visible amount of polysaccharides in DNA samples. In addition these authors suggested the use of fresh and young tissues, and the novelty of this procedure was that chloroform:phenol extraction or cesium chloride centrifugation was no longer needed.

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Table V. Current available DNA extraction protocols for cacti species including *Opuntia* spp

References	***Species analyzed***	***Protocol basis***		***Yield***	***Application***
(*101*) **Tissue source:** Cladodes	85 different species; some subfamilies: • Pereskioideae • Opuntioideae • Cactoideae	Two main extraction steps: 1) CTAB buffer 2) STE buffer Lysis with 20% SDS Precipitation with 5M Potassium acetate Centrifugation	Filter with Miracloth Precipitation with 7/10 vol isopropanol Centrifugation Pellet washing and drying Re-suspension in TE buffer	Subfamily Pereskioideae • *Pereskiopsis* 35(35) ng/µL SubfamilyOpuntioideae • *Opuntia* 540(250-2000) ng/µL Subfamily Cactoideae • *Coryphanta* 558 (363-933) ng/µL	Amplification of RAPD fragments from the isolated genomic DNA
(*103*) **Tissue source:** Roots	***Hylocereus :*** *H. undatus; H. polyrhizus; H. costaricens; H. ocamponis; H. purpusii* ***Selenicereus:*** *S. megalanthus; S. grandiflorus*	Extraction on ice, centrifugation Dissolve pellet with the same extraction buffer , centrifugation. Second extraction with CTAB buffer and 30% Sarkosyl. Extract with chloroform:isoamylalcohol Centrifugation Precipitation with isopropanol and sodium acetate solution, Centrifugation	Pellet washing with EtOH 75%, Dry and resuspend in TE, RNAse treatment, then phenol:chloroform extraction. Precipitate with EtOH 100% and sodium acetate, Centrifugation, followed by pellet washing with75% EtOH, air drying Re-suspension in 50-100 µL TE buffer	10-20 µg DNA/g fresh roots.	Amplification in RAPD reaction with reproducible profiles

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References	***Species analyzed***	***Protocol basis***		***Yield***	***Application***
(*100*) **Tissue source:** Cladodes	*Cleistocactus sp* *Echinocereus sp* *Nopalea cochellinifera* *O. albicarpa* *O. ficus-indica* *O. humifusa* *O. robusta* *Stenocereus pruinosus*	Extraction with CTAB buffer containing β-mercaptoethanol (prior to use), extraction with chloroform/isoamyl alcohol, Filtering step through Miracloth, Separation buffer extraction, Centrifugation Pellet dissolved in 1M NaCl, DNA precipitated with 2.5 vol EtOH 100%	DNA strands were spooled and air dried. Finally re-suspended in 100-300 μL TE	29-153 μg DNA /g tissue for all cacti plants evaluated	PCR amplification
(*104*) **Tissue source**: 100 mg of cladode	*O. ficus-indica* (L.) Mill var Gigante	Kit Amersham Pharmacia Biotech (Nucleon Phytopur for Plant DNA extraction) with some modifications to the protocol provided. An internal slice of the sample was used, the cuticle was removed avoiding the inclusion of areolar meristems, if the mucilage was higher than 4%, PVP-40000 was included in reagent 1.	Centrifugation. The supernatant (SN) was extracted with chloroform:isoamylalcohol SN was mixed with isopropanol and centrifuged. The pellet was washed with 70% EtOH (2X), dried and dissolved in sterile water.	100 μg DNA/g fresh weight	Amplification in RAPDs

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Table V. (Continued). Current available DNA extraction protocols for cacti species including *Opuntia* spp

References	*Species analyzed*	*Protocol basis*	*Yield*	*Application*
(*5*) Tissue source: seeds	*O. megacantha* Salm Dyck; *O. ficus-indica* (L.) Mill; *O. albicarpa* Scheinvar; *O. joconostle* F.A.C Weber; *O. streptacantha* Lem; *O. robusta* H.L. Wendl; *O. hyptiacantha* F.A.C Weber; *O. lasiacantha* Pfeiff; *O. undulate* Griffith; *O. cochinera* Griffith	DNA extracted from a mixture of seeds from three ripened prickly pear fruits per plant using a modified version of (*100*) based on a CTAB extraction protocol	Sufficient amount of DNA, although concentrations were not reported in this article	RAPD analysis
(*37*)	62 Opuntia species Some examples: *O. ficus-indica* (L.) Mill; *O. megacantha* Salm-Dyck; *O. streptacantha* Lem	DNA was isolated from the outermost layer of mature cladodes using Isolate Plant DNA Mini Kit (Bioline, London, UK)	Not reported in the article	Microsatellite analysis

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A DNA isolation technique for cacti such as *Hylocereus* and *Selenicereus* was developed (*103*). The abundant polysaccharides present in these species interfere with DNA isolation. Therefore, these authors decided to employ the roots of these plants as the source tissue due to the lower viscosity of the extracts relative to that of other tissues. Hence, the DNA samples prepared by this method could consistently be amplified in the RAPD reaction and gave reproducible profiles (Table V). The success of this protocol was attributed to the tissue extraction with CTAB coupled with the use of high salt concentration (4M NaCl), that suppressed co-precipitation of polysaccharides and DNA. Finally these authors suggested that this protocol could be employed in other plant tissues as well as in other cacti species (such as Opuntia).

Also, during RAPD analysis of Opuntia cultivars it was employed a commercial kit for DNA extraction which was used with some modifications in the protocol provided (Amersham, Pharmacia Biotech) (*104*). The distal part of cladodes of comparable age was cut, and an internal slice of the sample was taken for analysis to avoid contamination and inclusion of lysed cells. As a result, DNA yields were achieved typically ranging between 4.6 and 27 µg/g fresh tissue, with the tendency of upper values by increased routine in the procedure. A 3-week old cladode of an additional accession of *O. ficus-indica* yielded 100 µg DNA/ g fresh weight (Table V). The extraction method is called a micro-method, considering the small amount of plant material used in comparison to existing methods for DNA extraction of cactus plants. Additionally, the yield of DNA calculated per g of fresh tissue was very high. Extraction of 100 mg of tissue allows up to 1000 RAPD analyses.

Luna-Páez *et al.* (*5*) reported that the excess of polysaccharides present in nopal cladodes limits the use of molecular markers since DNA usually binds to these compounds, making it very difficult to analyze them by conventional techniques such as PCR. Hence, these authors decided to obtain DNA from seeds because these tissues lack polysaccharide compounds (Table V). Also, Caruso *et al.* (*37*) isolated DNA from the outermost layer of mature cladodes from 62 wild and cultivated genotypes belonging to 16 Opuntia species, using the Isolate Plant DNA Mini Kit (Bioline, London, UK) while analyzing the use of microsatellite markers that could help to asses genetic diversity among cultivated genotypes of *O. ficus-indica* and their relation with some other Opuntia species (Table V).

RNA

A protocol for the isolation of functional RNA from cactus fruits was developed (*105*). Blanca cristalina cactus fruits (*Opuntia* spp) at green or ripe stages were used for this study. In general, the optimized procedure consisted of applying a first step of extraction followed by a precipitation step with 5M potassium acetate and absolute ethanol; next a phase separation step was included; and then, the supernatant was recovered and re-extracted. Again, the supernatant was recovered in order to be re-extracted. According to these authors, the quality of the RNA extracted allowed for reverse transcriptase PCR (RT-PCR) amplification and cloning of the polygalacturonase cDNA from prickly pear. Also this method was evaluated in mango fruit (*Mangifera indica* L.) and pitahaya (*Hylocereus* sp).

Silva-Ortega *et al.* (*106*) while evaluating the expression of the *p5cs* gene under saline stress and its effect on proline accumulation in the cactus pear, isolated total RNA from *O. streptacantha* cladodes with the RNeasy Plant Mini Kit (QIAGEN) as recommended by the manufacturer, in order to use this for cDNA synthesis. The extraction proved to be successful in this plant and the addition of extra steps besides the manufacturer′s recommendations is not described. However, no problems related to the mucilage or polysaccharide content were reported by them when using this procedure.

A total RNA isolation protocol was developed and found that this procedure was a reliable method to detect for the first time sRNAs in nopal (*6*). In brief, three different total RNA isolation protocols were compared: Trizol ®, CTAB and LiCl; all of them evaluated on cladode and floral buds from *O. robusta*. According to these authors the LiCl method produced the best RNA quality and a positive relationship between the quality and yield of low molecular weight RNA and total RNA was observed. Thus, an effective isolation technique was obtained suitable for sRNA extraction from polysaccharide-rich material such as cactus, agave, banana and tomato tissues which was also evaluated in less complex plants such as arabidopsis and tobacco. Even more, the low molecular weight RNA was successfully utilized for Northern blot analysis which is important for downstream applications such as cloning and sequencing of sRNAs.

Protein Extraction and Proteomics

Protein extraction protocols are very important in plants in order to elucidate plant enzymatic functions or protein profiles, and now they are highly useful for proteomic analysis. Recently, Guevara-Figueroa *et al.* (*43*) evaluated the protein content of commercial and wild varieties of nopal (*Opuntia* spp.) for proximate composition. Total protein was quantified using the Bioassay kit (Biorad, Hercules, CA, USA). Protein pellets were re-suspended in Laemmli buffer and proteins were separated in 15% SDS-PAGE at 20 mA/gel. Protein extraction is more difficult from cladode samples but these authors have developed a methodology to allow obtaining clean protein samples (*107*). The band observed around 50 kDa in all varieties may correspond to the Rubisco large subunit reported in other plants (*108*).

In the post-genomic era, research starts to focus gradually from genes and genomes to proteins and proteomes for functional analysis (*109*). With this in mind, the conditions for proteomic analysis in prickly pear were developed (*110*). Therefore, three different morpho-species with different ripening behavior (early, intermediate and late ripening) were chosen. The conditions for the differential expression analysis trough 2-D electrophoresis were established. The proteins were isolated from three different materials; the protein concentration increased through ripening which indicates an active protein synthesis. The main proteins were found in a 5-7 pH range and with a 20-80 kDa of molecular weight. Around 1,000 proteins were detected at the ripe stages in all materials. Some differentially

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expressed proteins were isolated and purified; their comparison in data bases showed identity with proteins associated to fruit ripening, such as fatty acid synthesis, anthocyanin synthesis and photosynthesis; the final goal was to identify the specific function for the peptides, which will help to identify the differences between these materials and with other fruits, and to understand fruit ripening through the proteomic maps of each fruit (*110*). The analysis and expression of the proteins may help to understand the *Cactaceae* biology, through the identification of the proteins and their comparison with other plant sequences (*109*).

Enzyme Isolation and Characterization in the Opuntia Genus

The isolation, purification and characterization of two termostable endo-1,4-β-D-glucanase forms from *O. vulgaris* was reported (*111*). Cladodes were chopped and homogenized to prepare a 20% w/v cladode homogenate, which was centrifuged to obtain a clear supernatant used as the enzyme source. The endoglucanase activity of the enzyme source was measured by the dinitrosalicylic acid method. The specific activity of the enzyme was established in relation to the total protein content of the enzyme source determined by the method. The purification step was done as follows: cladode homogenate supernatant was taken for solid ammonium sulfate precipitation to a final concentration of 80% at room temperature. The protein precipitate was dialyzed and the adsorbed material was eluted with a linear gradient of sodium chloride in the range of 0-1 M. Protein content and enzyme activity of the collected fractions were determined at 280 and 540 nm, respectively. The active fractions, corresponding to the peak of endoglucanase activity, were combined and loaded onto a Sephadex G-100 (21 x 0.5 cm) column.

Ravikumar *et al.* (*112*) reported the purification and characterization of a novel thermostable xylose isomerase from *O. vulgaris* Mill. Thermophilic xylose isomerase from the xerophytic eukaryote *O. vulgaris* can serve as a good alternative source of enzyme for using in the production of high fructose corn syrup. The existence of two temperature stable isoforms having optimal activity at temperatures 70°C and 90°C, respectively, was reported. These isoforms were purified to homogeneity using column chromatography and SDS-polyacrylamide gel electrophoretic techniques. Only the T_{90} isoform was subjected to full biochemical characterization thereafter. The purified T_{90} isoform was capable of converting glucose to fructose with high efficiency under the assay conditions. The enzyme at pH 5.7 exhibited a preference to yield the forward isomerization reaction. The melting temperature of the native enzyme was determined to be 90°C employing differential scanning calorimetery. Thermostability of the enzyme was established through temperature-related denaturation kinetic studies. It is suggested that the thermostability and the wide pH activity of this eukaryotic enzyme will make it an advantageous and dependable alternative source of catalytic activity in the high fructose corn syrup sweetener industry.

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Biotechnological Applications

In Vitro Tissue Culture Methods

Under certain physical and chemical conditions plants can achieve *in vitro* organogenesis, which constitutes the process by which *de novo* organs such as buds, roots or shoots can be obtained from cultured tissues (*113*). Exogenous plant growth regulators (PGR´s) such as auxin and cytokinin must be balanced in the culture media, which is essential for *de novo* organogenesis (*114*, *115*). Whole plant regeneration may be achieved through either somatic embryogenesis or organogenesis (direct or indirect) (*8*). Micropropagation has been successful in different cacti genera (*Cereus, Equinocereus, Ferocactus, Mammillaria* and *Opuntia* among others) (*7*). During the last two decades, several propagation systems for the Opuntia genus have been described (*8*).

Micropropagation

Early reports in this matter started with Escobar et al. (*116*) who established the micropropagation of *O. amyclaea* by means of axillary proliferation. The effect of different concentrations of 6-bencilaminopurine (BA) on shoot development of explants sectioned transversely or longitudinally was determined after 25 days of culture. Therefore, according to these authors the best BA concentration was 10^{-5} in both types of explants, as evidenced by the development of shoots from 62 and 100% of the pre-existing axillary buds transverse and longitudinal sections respectively (Table VI). Axillary proliferation was evident in explants after 8 days in culture. According to these authors, the use of higher concentrations inhibited the number of axillary shoots. It was also noted that in the absence of BA the number of axillary buds was reduced significantly. When axillary shoots were evaluated for rooting, in different IBA (indole-3-butyric acid) concentrations, roots appeared in all transplanted shoots after 7 days in the auxin containing media. Interestingly, exogenous auxin was not so necessary for root differentiation since roots were formed even in the absence of auxins.

Also, Juarez and Passera (*117*) evaluated the *in vitro* propagation of *O. ellisiana* Griff and its acclimatization to field conditions. This plant is one of the slowest growing of all spineless *Opuntia* species; however, it exhibits high water use efficiency (162 kg H_2O /kg dry matter). The main goal of this study was to achieve massive propagation of *O. ellisiana* by *in vitro* culture of areoles (Table VI). The culture medium with BA and IBA, under a 16-h photoperiod, showed the highest percentage of areole shooting (100%) along with a shoot growth rate, thus being the most efficient culture medium for the optimal multiplication of this plant material. After transplant, plantlet survival was 100%, and after 7 months of culture a total of 1200 plantlets was obtained.

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Table VI. Plant *in vitro* tissue culture techniques developed for the Opuntia genus

Tissue culture technique	*Plant growth regulators combinations*	*Opuntia cultivar*	*Explants source*	*Reference*
Micropropagation	BA, IBA	*O. amyclaea*	Axillary buds	(*116*)
	BA, IBA	*O. ellisiana Griff*	Areoles	(*117*)
	BA, GA_3, IBA	*Opuntia spp* Milpa Alta, Villanueva and Blanco sin Espinas	Cladode	(*7*)
	GA_3, BA, DAP	*O. lanígera* Salm-Dyck	Areoles	(*119*)
Somatic embryogenesis	BA, IAA	*O. ficus-indica*	Cladodes	(*120*)
	2,4-D, kinetin, ABA	*O. ficus-indica*	Zygotic embryos	(*121*)
	picloram, BA	*O. ficus-indica* Gigante	Young cladodes	(*123*)
Indirect organogenesis	2,4-D, picloram, BA, kinetin, casein hydrolysate	*O. ficus-indica*	Cotyledons and hypocotyls	(*124*)
	2,4-D, BA	*O. ficus-indica* Blanco sin Espinas	Cladodes	(*8*)

2,4-D: dichlorophenoxyacetic acid; BA: bencilaminopurine; picloram: 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid; GA_3: giberellic acid ; IBA: indole-3-butyric acid; IAA: indole-3-acetic acid; DAP: 6-(γ,γ-dimetylalilaminopurine); ABA: abscisic acid; kinetin: 6-furfurylaminopurine.

Plant regeneration of three main cultivated materials from Mexico, namely Milpa Alta, Villanueva and Blanco sin Espinas used for human consumption, was achieved (*7*). *In vitro* obtained shoots were used as source of secondary explants for propagation experiments. Explants were cultivated during 15 days in MS (Murasigue and Skoog basal salts) (*118*) medium supplemented with different BA concentrations and after that they were transferred to hormone-free MS medium (see Figure 2).

The results showed that all materials respond at 0.5 µM BA (Table VI). Regarding root formation media, the best response was registered at 0.5X MS medium and 5.5 µM IBA. Accordingly, the capacity to form new buds was influenced by BA concentration. In brief this constituted the first report of a regeneration system for several Opuntia genotypes used as vegetables sources for human consumption.

The micropropagation of the ornamental nopal variety *O. lanigera* Salm-Dyck was developed (*119*). They evaluated the effects of sprayed GA_3 (giberellic acid) after transplantation to *ex vitro* conditions. Axillary shoot development from isolated areoles was established. Different explants orientations, type of cytokinin and their concentrations were evaluated for the shoot proliferation stage.

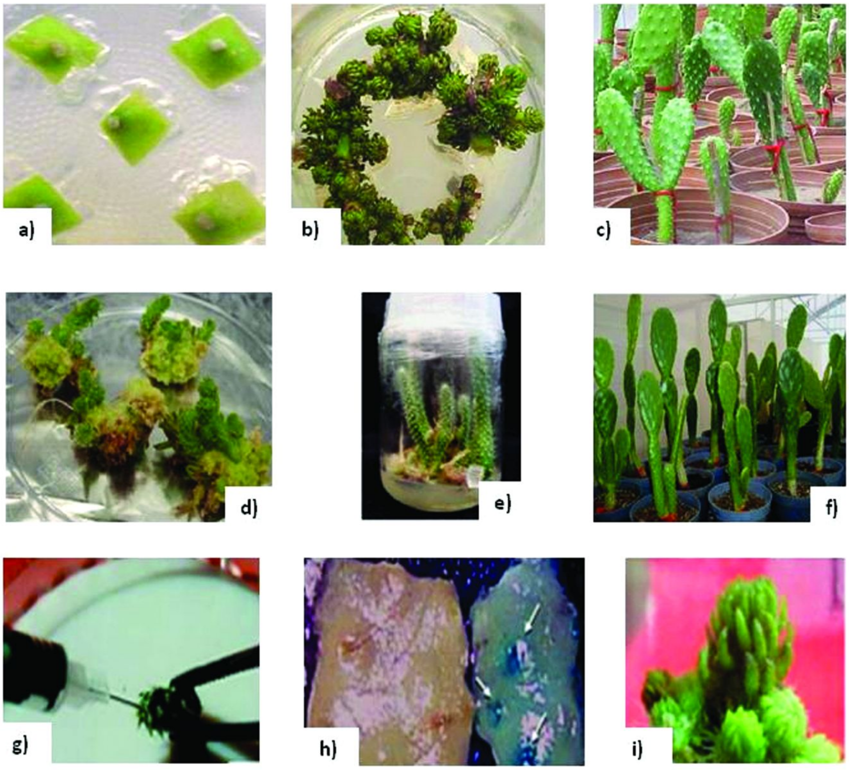

Figure 2. Nopal in vitro plant cell tissue culture and transformation techniques developed in our laboratory. a) to c): micropropagation of nopal (Reproduced with permission from reference (7). Copyright 2005 Springer.); d) to f): indirect organogenesis protocol (Reproduced with permission from reference (8). Copyright 2011 Elsevier.); g) to i): Agrobacterium tumefaciens genetic transformation of nopal. (Reproduced with permission from reference (130). Copyright 2006 Springer.)

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The effects of GA_3 on plant growth were determined by spraying a series of increasing concentrations. In general, after 42 days of culture, significantly higher shoot length was achieved with DAP [6(γ,γ-dimetilalilaminopurine)] compared to kinetin and BA (Table VI). Exogenous application of GA_3 after plantlet acclimatization on glasshouse conditions increased spine-hair (developed from areoles in young plants) length as part of short-term effects. However, significantly higher values were obtained in plantlets treated with 300 ppm of GA_3 when compared with the rest of treatments. At the end of the study, the most important long-term effect produced by GA_3 was the suppression of total shoot growth. The protocol may be used in commercial exploitations to regenerate many plantlets and produce healthy plants with better ornamental characteristics and higher commercial value.

The effect of different media on the *in vitro* growth of cactus (*O. ficus-indica*) explants was evaluated (*120*). Two different media were evaluated on cladode explants from *O. ficus-indica*, the first containing only BA and the second supplemented with BA and IAA (indole-3-acetic acid). According to these authors, the difference in concentrations of BA in both media may have played a role in neutralizing whatever effect the two different media may have exerted on the shoot length and emergence. In general, low levels, or no auxin are required in combination with moderate to high levels of cytokinin for cactus axillary shoot proliferation. Accordingly, no great differences were observed between the use of any of the two media evaluated, thus the authors suggest that this can be attributed to the short period of time used to evaluate the response of the explants (Table VI).

Somatic Embryogenesis

Pinheiro da Costa *et al.* (*121*) studied the induction of embryogenic globular structures in *O. ficus-indica*. The seeds of *O. ficus-indica* were disinfested and mechanical isolation of zygotic embryos from the seeds was performed under sterile conditions (Table VI). Embryos were cultured for 4 weeks in liquid modified B5 medium (*122*), on a shaker at 100 rpm, containing various concentrations of 2,4-D (dichlorophenoxy acetic acid) as the only source of PGR or in combination with kinetin (at different concentrations) or 10^{-4} mol/L abscisic acid (ABA). After 4 weeks of primary culture in the presence of PGR′s, the germinated embryos were sub-cultured at 4 week intervals by applying the same basal medium without any PGR. Germination rate and development of the embryos in the nutrient medium were influenced by applied PGR′s and light. The presence of 10^{-4} mol/L ABA alone or in combination with 0.45 mol/L 2,4-D and darkness reduced the rate of germination and postponed the development of the zygotic embryo significantly. The highest rate of germination and a strong formation of the root as well as of the cotyledons were observed in the absence of any growth regulator. In the presence of lower concentrations of 2,4-D in the medium, development of the root was reduced or inhibited, malformation of root and shoot was frequently observed and preferably at the regions of the cotyledons, globular structured callus growth was obtained. However, microscopic analysis revealed that these globular structures did not show the

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typical morphological characteristics of embryogenic globular structures. The putative globular structures found in these callus cultures of *O. ficus-indica* could not be separated easily from the originating tissue and the structures were fragile and did not display a distinct outer layer to give a well-defined and stable globular shape. Nevertheless, by adding kinetin (0.91 x10^{-6} or 9.1 x10^{-6} mol/L) to the medium with 0.45 x10^{-6} mol/L 2,4-D induction of globular structures were achieved, which demonstrated the typical morphological characteristics of embryogenic material, and could be observed about 3 to 4 weeks after inoculation of the isolated zygotic embryos emerging preferably at the cotyledonary tissue. The higher concentration of kinetin gave more reliable results, during primary culture of 4 weeks; a higher number of zygotic embryos with induced somatic embryos were obtained.

The somatic embryogenesis and plant regeneration in *O. ficus-indica* (L.) Mill (Cactaceae) was reported (*123*). The explants induction of somatic embryogenesis (SE) were shoot apices isolated from *in vitro* grown shoots of *O. ficus-indica* cv Gigante (Table VI). The induction medium was supplemented with picloram (4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid). For embryo maturation and germination, the somatic embryos were transferred to maturation media containing also picloram plus BA. Incubating under dark conditions of shoot apices lacking spines was crucial for the induction and histo-differentiation of the somatic embryos produced. The embryos appeared by budding on the surface of the explants after 2 weeks as globular-shaped structures, having a white opaque color; within 30 days, these globular embryos differentiated into more advanced developmental stages. However, the attempts to mature these embryos in medium lacking PGRs only resulted in callus growth on embryos clusters without any plant recovery. Embryo maturation and plant recovery was observed by transferring these clusters to medium containing picloram and BA. In brief, the somatic embryogenesis conversion frequency was 12.5%.

Indirect Organogenesis

The establishment of callus and cell suspension cultures of *O. ficus-indica* was published by Llamoca-Zarate *et al.* (*124*). Calli cultures were initiated from cotyledon and hypocotyls sections of 21 day old light grown seedlings (Table VI). Seedlings were disinfected and hypocotyls and cotyledon sections were then transferred to Petri dishes containing 30 mL of semi-solid medium, consisting of full strength MS salts and vitamins, 30 g/L sucrose, 8 g/L agar and supplemented with different combinations and concentrations of PGR´s (2,4-D, kinetin, and picloram). The cultures were incubated in the dark at 28°C. Calli were separated from explants and subcultured at 3 to 4 week intervals onto medium with 2.3 μM 2,4-D, 0.9 μM kinetin, 1.0 μM picloram and 400 mg/L casein hydrolysate, this medium was designated friable callus medium (FCM). Cell suspension cultures were initiated by shaking 35-40 g of friable calli at 150 rpm in the dark at 28 °C. The growth of friable callus was evaluated by measuring diameter and by fresh weight. Explants from cotyledons produced significantly more callus than those from hypocotyls. The MS medium supplemented with 0.9 μM kinetin, 2.3

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μM 2,4-D and 1.0 μM picloram produced calli with better growth properties than the other tested combinations of supplements. The soft friable and fast-growing calli were separated from non-friable callus and further propagated in FCM. The percentage of friable calli was 86%; only the friable aggregates were propagated. Soft calli contained mostly round and elongated cells with thin cell walls and large vacuoles. The best growth of calli was observed in the linear phase of growth between 14 and 21 days of culture. According to these authors, a pre-requisite for establishing friable callus of *O. ficus-indica* was the presence of picloram and casein hydrolysate in the culture medium, as well as the selection of white-yellow, soft calli.

An indirect organogenesis regeneration protocol for *O. ficus-indica* (L.) Mill cultivar Blanco sin Espinas was developed by Angulo-Bejarano and Paredes-López (*8*). Explant sections of 1 cm^2 from previously micropropagated prickly pear plants were cultured in MS basal medium supplemented with 20 different combinations of 2,4-D and BA (Table VI). The best calli induction and regeneration response were observed when an almost 1:1 combination ratio of 2,4-D vs BA was applied to the nopal explants. New buds were formed from regenerating calli after transferring them to MS medium supplemented with BA; shoot elongation and rooting conditions were achieved on medium devoid of PGR′s. Excellent acclimatization to greenhouse conditions was observed in all transferred plantlets, therefore no morphological differences were observed between the regenerated and mother plants (see Figure 2). Accordingly, the established protocol might be employed for plant regeneration after genetic transformation. This is the first report of this nature in *O. ficus-indica*, since the goal of most Cactaceae propagation studies are centered on micropropagation, especially in the Opuntia genus (*125*).

Genetic Transformation in Prickly Pear

Particle Bombardment

The transient gene expression of the *nptII* (neomycin phosphotransferase II) and *uidA* (β-glucoronidase) genes when bombarded over friable calli of *O. ficus-indica* cell suspensions was reported (*124*, *126*). For this purpose, friable calli sections were used as explants source and kept in osmotic medium 4 h after bombardment. The distance between the flying disk and the target tissue was 7.5 cm and the shooting pressure was adjusted to 1200 psi (Table VII). One hundred milligrams per liter kanamycin were used for calli regeneration. Transgenic friable calli were obtained on kanamycin containing medium after particle bombardment.

Foreign gene integration was confirmed by histochemical and photometric analysis for GUS activity and by PCR amplification of DNA with *uidA*-gene specific primers, *nptII* primers and *tet* gene primers. However, no stable integration of the transgene(s) was demonstrated, nor the full putative transgenic plantlet regeneration.

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Table VII. Plant genetic transformation techniques applied in the Opuntia genus

Opuntia cultivar	*Plant transformation system*	*Strategies*	*Regeneration*	*Reference*
O. ficus.indica	Particle bombardment	Cell suspensions bombarded with *nptII* and *uidA* containing plasmids. 7.5 cm flying disk to target tissue plus 1200 psi pressure	Regeneration was observed in 100 mg/L kanamycin. PCR and gus fluorescence were observed. No stable regenerant plants	(*126*)
O. ficus-indica	Particle bombardment	Shoot apical meristems bombarded with plasmid containing uidA and *nptII* genes	7.5 cm plus 1200 psi. Non stable transformation was reported	(*127*)
O. ficus-indica	Particle bombardment	Shoot apical meristems bombarded with atahas and *uidA* genes	Bombardment conditions used reported by (*129*). PCR and X-gluc staining positive. No stable gene integration was demonstrated	(*128*)
O. ficus-indica	*Agrobacterium tumefaciens*	Nopal cladode explants from previous in vitro propagation. *A. tumefaciens* GV2260 harboring the pBI121 plasmid with *npt II* and *uidA* genes	Stable gene integration was demonstrated by PCR and Southern blot hybridization.	(*130*)

uidA: β-glucoronidase; nptII: neomycin phosphotransferase II; Atahas: acetohydroxyacid synthase; X-gluc: 5-Bromo-4-chloro-3-indolyl-β-D-glucoronide sodium salt.

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In a second approach Llamoca-Zarate *et al.* (*127*) reported the biolistic mediated transient gene expression; this time on shoot apical meristems of the *O. ficus-indica*. Shoot apical meristems (0.5-0.8 mm) were excised aseptically and transferred to a BA and GA_3 containing medium where they were kept during 12-16 h before bombardment. The plasmids employed were pFF19G which contains the *uidA* gene controlled by the CaMV35S promoter, pNG which contains the *uidA* and *nptII* genes under the control of the CaMV35S promoter and pPARGUSH which contains the *nptII* gene under the control of the nos (nopaline synthase) promoter and the *uidA* gene under the control of the par promoter (Table VII). The particle bombardment conditions employed were: tungsten particles of 1.3 µm, the distance between the flying disk and the target tissue was 7.5 cm, and the shooting pressure was adjusted to 1200 psi. The results of the transient expression experiments indicated successful delivery of foreign DNA into apical meristem cells of the prickly pear. Cells of the central zone of the meristem transiently expressed the GUS gene; meristem cells expressed the reporter gene at higher frequency than surrounding tissue. Control meristems bombarded with uncoated particles did not show any blue spots; thus demonstrating the lack of endogenous GUS activity in *O. ficus-indica* tissues. No differences in the frequency of expression of the transgene were observed when the meristems were bombarded with different plasmids. However, no stable transformation of these explants was reported.

Later on, Cruz *et al.* (*128*) reported another approach for particle bombardment gene delivery to shoot apical meristems of *O. ficus-indica*. They reported the bombardment of these explants with the pGA1 plasmid which contains the *gus* gene and the *Atahas* gene (coding for acetohydroxyacid synthase, E.C 4.1.3.18), conferring resistance to imazapyr, a herbicidal molecule that concentrates in the apical meristematic region of the plant (Table VII). The bombardment was done according to Aragão et al. (*129*); multiple shooting was induced by transferring the explants to induction medium immediately after bombardment. As soon as the herbicide-tolerant explants were 3-6 mm in length, a 1 mm long section was removed from the base for the histochemical analysis. For PCR analysis, DNA was isolated using CTAB methods, and the reaction was carried out to detect the *Atahas* coding sequence. The transformation frequency obtained by the system reported here was 4.1%. Nevertheless, no further molecular evidence was provided in order to establish and prove the transgenic nature of these explants (Southern blot for instance).

Agrobacterium Tumefaciens

Silos-Espino *et al.* (*130*) reported the genetic transformation of an elite prickly pear cactus namely, *O. ficus-indica* L., cv Villa Nueva by *Agrobacterium tumefaciens*. It was started with direct bacterial infection by using a hypodermic syringe to the meristematic tissue termed areoles using the binary pBI121 vector for transient and stable expression assays (Table VII). However, these authors emphasize that to solve in part the possibility of having chimeric plants because of the complex organization of the shoot apex itself, resistant structures were

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dissected from the original explants to a fresh selective medium every 2 weeks for eight additional weeks; after that, selected explants were transferred onto fresh regeneration medium supplemented with kanamycin, where all of them responded positively for growth and development of a whole plant. Transgenic plants were obtained by selection with kanamycin. Transient and stable GUS activities were monitored on kanamycin-resistant shoots and regenerated plants, respectively. Genetic transformation of regenerated plants growing under selection was demonstrated by PCR and Southern blot analysis. Transgene copy number in the genome of transgenic plants ranged from two to six, while the transformation frequency obtained by the system reported by them was of 3.2%. Up to now, this constitutes the first and solely report of this nature for the Opuntia genus, and therefore this method may be useful for routine transformation and introduction of several important genes in prickly pear cactus.

Food Industry Applications

During the first half of the 20th century, food scientists were focusing on undernutrition and strategies to modify foods to correct nutritional deficiencies; therefore, throughout the last decades a marked increase towards the use of natural products such as fruits, herbs, oilseeds and vegetables as functional foods and sources for nutraceutical antioxidants and functional foods has emerged. Accordingly, these natural antioxidants can be used in the food industry and much available evidence suggests that these substances may exert their antioxidant effects in human health. Therefore, functional foods may contain one or more ingredients have been manipulated to enhance their contributions to a healthful diet (*131*).

In this sense the use of nopal for human consumption is viable for various countries around the globe, Mexico being the first consumer of this plant using both cladodes and fruits. In view of so many reports indicating the high nutritional value and potential nutraceutical activities exerted by this cactus plant it is not surprising that in recent years it has been incorporated as an extra ingredient in new healthier food alternatives.

The beneficial effects on human health of dietary fiber are well documented including natural and synthetic dietary fiber. On the other hand, recently more sources of fiber are being evaluated in order to provide functional properties such as water holding capacity and improvement in mechanical and rheological properties, among others (*132*). In this sense, Guevara-Arauza *et al.* (*133*) evaluated the use of biofunctional activity of tortillas and bars enhanced with nopal, providing a preliminary assessment of functional effect after intake on the oxidative status in healthy volunteers. The total phenolic content was increased with the addition of nopal in both tortillas and bars. Total phenol content between different formulations of bars and tortillas can be due to different interactions among phenolic compounds and other formulation components. The addition of nopal to the formulation of tortillas and bars increased the dietary fiber, ash, but not the protein content. In brief, the consumption of tortillas supplemented with nopal daily during 21 days, improved the oxidative status of healthy humans.

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In addition, levels of glucose, cholesterol (total and LDL) and triglycerides decreased significantly.

According to what we have reviewed in previous sections of this chapter, nopal plants exhibit high polyphenol contents among other nutraceutical compounds. However, food industry process may affect their content. The effect of air flow rate on total polyphenol content and antioxidant capacity of convective dried cactus pear cladodes (*O. ficus-indica*) was evaluated (*134*); the authors suggested that the composition of extracts from *O. ficus-indica* cladodes were influenced by the air flow rate. The total phenol content, flavonoids and flavonols are lower when the air flow rate was higher. The antioxidant capacity was not linearly related to the concentration of polyphenols. LDL inhibition only showed differences in function of the concentration. The extracts were more effective in inhibition of LDL oxidation that in the function of the radical scavenging activity.

New applications for different plant parts of nopal are emerging. Sáenz *et al.* (*135*) reported the microencapsulation of bioactive compounds of the pulp and ethanolic extracts of cactus pear (*O. ficus-indica*); they were encapsulated either with maltodextrin or inulin. In brief, betacyanins degradation rate in pulp microcapsules occurred at the same rate in both encapsulating agents, whereas in the ethanolic extracts it was significantly lower in inulin than in maltodextrin, showing better performance of inulin. Indicaxanthin in all systems studied showed a slow degradation during storage at 60 °C and more stability than betacyanin. On the other hand, an increase of stability in phenolic compounds was observed in pulp and ethanolic extracts encapsulated with inulin or maltodextrin during storage at 60 °C. According to these authors the microcapsules produced with this method represent an interesting food additive for incorporation into functional foods, due to both the presence of antioxidants and red colorant.

Medina-Torres *et al.* (*136*) evaluated the influence of different drying methods on mechanical properties of nopal (*O. ficus-indica*). In general, convective and osmotic drying as well as a combination of both methods were performed. The osmotic drying moisture content was correlated with glucose content. Dehydrated nopal by convective drying exhibited more cohesiveness than samples treated by osmotic drying. However the samples treated by osmotic drying were more elastic. These results can be used in the food industry mainly for nopal preservation through dehydration.

Betalains are the pigments responsible of red color in prickly pear fruits and they are even more interesting for their antioxidant capacity associated to health benefits. Therefore, the potential use of betacyanin pigments from *O. stricta* fruit juice as natural red purple food colorants was evaluated (*137*). A powder food colorant was obtained by concurrent spray drying from *O. stricta* fruit juice. This product showed high color strength, which was stable when stored at room temperature for one month, and it was successfully applied in two food model systems: yogurt and a soft drink. Food presented a vivid red-purple tonality very attractive for consumers, which was maintained after one month under refrigeration at 4°C.

In brief, the nopal cladodes and fruits have a very strong potential for a wide use in food and nutraceutical products in view of their composition and attractive sensory properties. Massive production of the plant cladodes and fruit has been

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intensively studied as well in the last decades. Additionally, this plant has a very strong agronomic potential to be cultivated under optimal and not so optimal environments. Thus, this Mexican and Mesoamerican food deserves a better future, not only in this geographical region but also in other countries of the world.

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Chapter 10

Microstructure of Mature Green Mexican Vanilla Pods *Vanilla planifolia* (Andrews) by Microscopy Techniques and Digital Image Analysis

A. P. Tapia,[1] D. I. Téllez,[1] M. J. Perea,[2] E. Ortiz,[3] and G. Dávila*,[1]

[1]Departamento de Graduados e Investigación en Alimentos, Escuela Nacional de Ciencias Biológicas-IPN, Carpio y Plan de Ayala, Col. Santo Tomás 11340, México, D. F
[2]Centro de Nanociencias y Micro y Nanotecnologías-IPN, Unidad Profesional "Adolfo López Mateos" Luis Enrique Erro s/n. Zacatenco 07738, México, D. F
[3]Departamento de Morfología, Escuela Nacional de Ciencias Biológicas-IPN, Carpio y Plan de Ayala, Col. Santo Tomás 11340, México, D. F
***E-mail: gdavilao@yahoo.com**

Vanilla, which originated in Mexico, is a tropical orchid belonging to the family Orchidaceae. The green vanilla pods contain glucosyl precursors of aroma compounds. The aroma is released only after "curing", when the glucovanillin is hydrolyzed by glucosidase in the vanilla pods. This study describes the microstructure of mature green vanilla pods (*V. planifolia*) from Papantla de Olarte, Veracruz, Mexico. Nine structures were differentiated by stereomicroscopy, and light and environmental scanning electron microscopy: epicarp (EP), outer and mid mesocarp (OM, and MM), vascular bundles (VB), endocarp (EN), placentae (P), trichomes (TC), crystals of calcium oxalate (CR) and seeds (SE). The morphometric parameters of the cells identified in each structure such as area (A), perimeter (P), shape factor (SF) and Feret's diameter (FD) were quantified by digital image analysis (DIA) using light microscopy and thus defined the average size and approximate shape of the cells. Additionally, through the application of DIA to the micrographs captured with the stereomicroscope, five

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main compartments constituting a vanilla pod were identified and quantified as the percentage of the space occupied by each compartment at the pseudo-prismatic region of the pods: 1) EP, OM, MM and EN: 66.55%, 2) P: 16.09%, 3) SE: 14.42%, 4) TC: 2.90%, and 5) the intralocular interstitial cell-free region (locule): 0.04%. It is concluded that the use of DIA techniques allows a more accurate morphometric characterization and evaluation of the vanilla pod parameters.

Introduction

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The family *Orchidaceae Juss.* involves more than 800 genera and 25,000 species. The *V. planifolia* is native to the Mexican tropical forests; nowadays, it is cultivated in two regions: the north of Veracruz, and the north of Oaxaca (*1*). In accordance with vegetative characteristics, four morphological varieties of Mexican vanilla have been identified, among which mansa or fina, mestiza y de tarro stand out as the most common types (*2*). Two mansa sub-types, yellow and green, may be distinguished by their roots and the color of their leaves (*2*). The odor characteristics of vanilla are determined after a procedure called “curing” which is carried out from the green fruits, i.e. those fruits containing glycoside compounds at the time of harvest (*3*, *4*). The “curing” applied to the green mature pods aims to hydrolyze the glycosides and thereby the release of the compounds responsible of the vanilla’s odor (*5*, *6*). The “curing” process of pods lasts three months, although it could take up to six months depending on the source region and country; the procedure differs for every part of the world and also between authors (*7*). Anatomical works have been reported and analysed by using different microscopic techniques: optical (*8*), confocal laser (*9*), scanning and transmission electron (*10*). In such studies, the histological characterization of green mature vanilla pods has been performed as a general description of the cells from EP, OM, MM, EN and TC, as well as of P and SE. However, morphometric parameters such as A, P, FD and SF, obtained through DIA, have not been used to quantitatively describe the cells of the identified structural regions in the green mature vanilla pods.

The DIA is a series of reliable tools for obtaining quantitative data from complex structures such as cells and vegetal tissues (*11*); it is a recently developed technique involving the acquisition and digitalization of images captured by an optical device (photography or microscopy) coupled to a computer. The digitalization means the transformation of an image into a matrix of points which contain information about their position, lightness, color, etc (*12*); thereby, several measurements may be performed on the digitalized image, e.g. cell perimeter and area, cell counting, and color density. The information provided by the image allows for numerous applications of the DIA in food science and technology (*13*).

The DIA applied to micrographs includes four main steps (*14*, *15*): 1) image capturing, 2) image processing, 3) measurement of characteristic parameters, and 4) reconnaissance, classification and interpretation of the images. This particular method has the advantage of being a precise tool capable of producing quantitative data (*16*). This is important because in food science and technology there is often need of obtaining numerical information from images of biological materials (*17*, *18*). Only few studies report simultaneously both, images and numerical data of structures that could be indicative of certain functional properties. Although morphometric descriptions of cells and tissues have been reported before for other vegetable species (*11*, *15*, *19*, *20*), this type of analysis has not been conducted in Mexican vanilla spp.

By considering the above, the purpose of this investigation was to obtain morphometric data of the constituting cells of different identified structural regions of green mature vanilla pods Mexican *V. planifolia*. The spatial ratio for every identified compartment was also determined, in order to contribute to enhance the information found in the literature on their histological characterization.

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Materials and Methods

Biological Materials

The vanilla pods were supplied by the Mexican National Council of Vanilla Producers (CONAVAI). The samples were obtained in the Totonacan region of Papantla de Olarte, Veracruz (latitude: N 20° 27’, longitude: W 97° 19’) in December 2008. The pods were collected at their ripeness state (nine months after hand pollination) and transported to the laboratory in hermetically sealed plastic bags, which were further stored in a vacuumed iced container, for no more than 24 h after cutting.

Histological Characterization

The criteria proposed by Odoux et al. (*21*) were used to perform the histological characterization of the structural regions of vanilla pods. The biological material was transversally cut in the pseudo-prismatic region (in the middle of the pods) (*22*) in order to obtain the histological sections.

Observations under Stereomicroscope

The green mature vanilla pods were transversally cut by hand in sections of 5 mm thickness. From these samples, images were obtained with a stereomicroscope (Nikon SMZ 1500, Japan), coupled to a digital camera (Nikon DS- 2 Mv, Japan) and an interface system (Nikon DS-U2, Japan), connected to a computer (Hewlett-Packard-Compaq DC 5700). The images were captured by means of the software NIS-Elements F 2.30, with size 1600 × 1200 pixel^2 (1 pixel = 1.16 μm), using the RGB colour system, and stored with TIF format. A total of ten images of the transversal view of the pseudo-prismatic region were captured, corresponding to each of ten different vanilla pods.

Observations under Light Microscope

The fresh material was left in a mixture of 70% ethanol-glacial acetic acid-formaldehyde-water 50:5:30:15, for 48 h. The material was then embedded in paraffin and sectioned (14 µm thickness) by using a microtome (Minot, Leica). The colour agent was Safranin O-fast green. The sections were placed on a slide with synthetic resin (Entellan, Merck) and observed under a light microscope (Nikon, Japan). The images were captured using the RGB colour system, and the TIF format, with size 448 × 287 pixel^2. Three images were obtained per identified structure (EP, OM, MM, VB, EN, P, TC, CR, and SE) for each of ten different vanilla pods. As total, 20 measurements were performed for each identified structure. Data were reported as average values.

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Observations by Environmental Scanning Electron Microscopy

The material was transversally cut by hand, to obtain sections of 3 mm thickness, which were subsequently placed over metallic plates and observed under the environmental scanning electron microscope (ESEM XL30, Philips, USA). The images were captured in gray-scale resolution and stored with TIF format, with size 448 × 287 pixel^2. Similarly as for light microscopy, three images were obtained per identified structure (EP, OM, MM, VB, EN, P, TC, CR, and SE) for each of ten different vanilla pods. Twenty measurements were performed for each identified structure, and the corresponding average is reported.

Cell Morphometry and Calculation of the Spatial Ratio of the Compartments

The images captured by using the light microscope were analyzed, in order to evaluate cell morphometry in each structural region. The spatial ratio for each identified compartment in the pseudo-prismatic region, namely: 1) EP, OM, MM and EN; 2) P; 3) SE; 4) TC; and 5) intralocular interstitial cell-free region, was determined by DIA from the micrographs captured by using the stereomicroscope. The DIA was carried out by means of the software ImageJ 1.34 (NIH, USA). The micrographs obtained by light microscopy were firstly modified to gray-scale resolution. The elements of interest were selected by adjusting the threshold, and then the images were converted to binary mode. The binary images were then used to determine the morphometric parameters: A, P, SF and FD, of the cells in each identified structural region of the pods. The spatial ratio of the compartments was determined from the binary images as well; these images were segmented with the purpose of obtaining the percentage occupied by each compartment in the transversal cut of the pod.

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Results and Discussion

Histological Characterization

The green mature pods of *V. planifolia* cultivated in this Mexican region (Papantla de Olarte, Veracruz) are tricarpal capsules with parietal placentation, green, succulent, straight or slightly curved, slightly trigonal and lately dehiscence pods. These characteristics are consistent with the observations described by other authors (*2*). From the micrographs captured with the stereomicroscope, the following structures were identified: EP, OM, MM, VB, EN, P, TC and SE (Figure 1A); the CR was observed by using the light and environmental scanning electron microscopes (Figures 1E and 1F). These findings are similar to those reported by other authors (*21*) for green mature pods of *V. planifolia* cultivated in the Democratic Republic of Madagascar. The histological sections observed under light and environmental scanning electron microscopes allowed for identifying, from the outer to the inner zones of the pods, the epidermis with a coarse cuticle where the EP was observed next to several layers of parenchyma. The EP consists of a layer of flat cells (Figures 1C and 1D); the thickness determined by DIA was 0.07 mm, while the rest of morphometric parameters were: 1760 ± 14.24 μm^2 A, 182 ± 3.01 μm P, 0.59 ± 0.11 SF, and 74 ± 1.90 μm FD. Next to the EP, the OM was found to be constituted by several layers (3.00 mm, total thickness) of polyhedral parenchyma cells of different sizes; and by light microscopy (Figure 1C) it was possible to observe that OM cells had smaller thickness than the EP cells. The morphometric parameters used to describe the cells of this structural region are summarized in Table I. Under the light microscope, the nuclei inside the MM cells were markedly observed in the cutting plane, as well as the needle-shaped, randomly distributed CR (Figure 1E) which were detected also under the environmental scanning electron microscope (Figure 1F). The mesocarp has been described as a developed parenchymatous tissue constituted by cells that are capable to increase their volume, from the EP to the MM where the cells reach 200-300 μm size (*21*). The parenchymatous tissue of the MM observed under light and environmental scanning electron microscopes consisted also of polyhedral cells of different sizes (Figures 1E and 1F); the morphometric parameters of this structural region obtained by DIA are shown in Table I. Within the parenchymatous tissue of the MM, 18 VB were observed to be arranged in groups of three bundles each and in accordance with the three angles of the pod, as well as three smaller bundles allocated between the groups (Figure 1B). The cells forming the vascular bundles were polyhedral, polydispersed (different sizes), thick-walled cells, as observed under light and environmental scanning electron microscopes (Figures 2A and 2B); The DIA applied to the images captured by light microscopy revealed the following morphometric parameters of this structural region: 3061 ± 11.64 μm^2 A, 246 ± 2.94 μm P, 0.69 ± 0.10 SF, and 78 ± 1.36 μm FD. With respect to the arrangement of the 18 VB in the parenchyma (Figure 1B), the number of bundles identified was similar to that reported by other authors (*8*, *21*) for green mature pods of *V. planifolia* cultivated in Papantla de Olarte, Veracruz, Mexico and the Democratic Republic of Madagascar, respectively.

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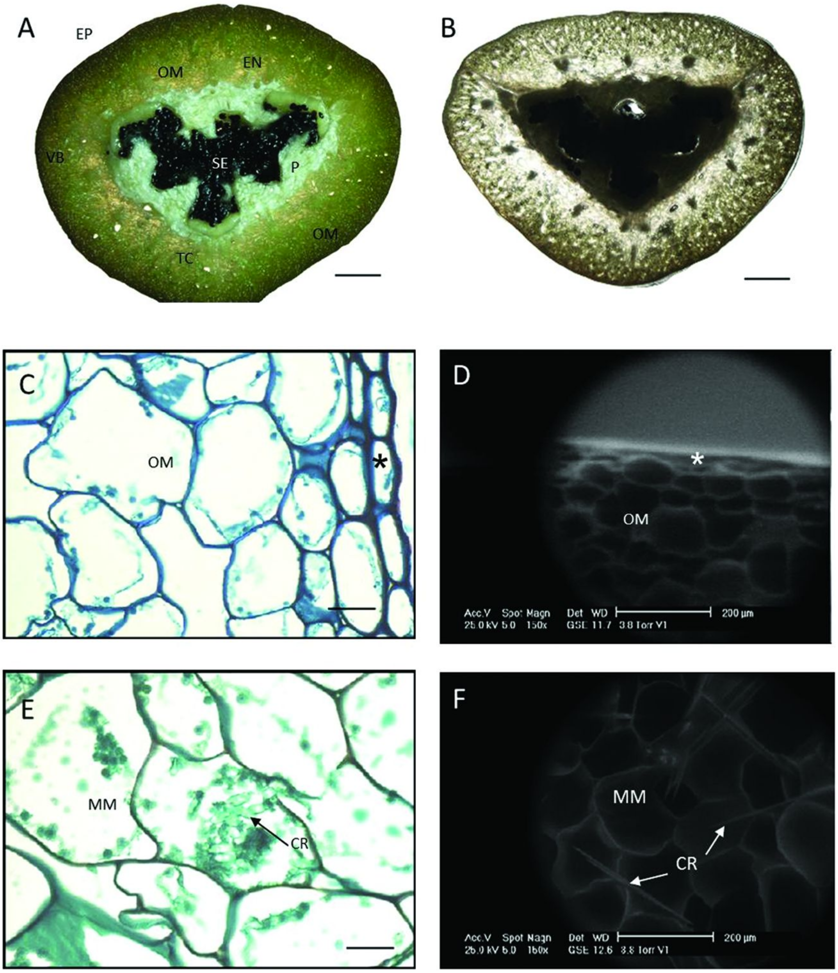
A
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MM
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F
MM
CR
Acc.V Spot Magn Det WD 200 µm
25.0 kV 5.0 160x GSE 12.6 3.8 Torr V1

Figure 1. A – F. Images corresponding to the transversal cut of a green mature pod (capsule) of V. planifolia Andrews; A and B, observations under a stereomicroscope: A, the different structural regions can be observed, and B, 18 vascular bundles, as observed; C and E, observations under a light microscope using Safranin O-fast green staining; D and F observations with environmental scanning electron microscope, it is possible to distinguish the epicarp (or EP), outer mesocarp (OM), mid mesocarp (MM), calcium oxalate crystals (CR), endocarp (EN); vascular bundles (VB); placenta (P); seeds (SE); trichomes (TC). The scale values are 2 mm (for A and B), 50 µm (for C and E), and 200 µm (for D and F).*

Table I. Morphometric parameters of the cells of OM and MM of the pods

Structural region	*A (μm^2)*	*P (μm)*	*SF*	*FD (μm)*
OM	9272 ± 32.37	443 ± 5.75	0.59 ± 0.15	133 ± 2.77
MM	10038 ± 20.80	432 ± 3.07	0.65 ± 0.08	146 ± 1.65

A: area; P: perimeter; SF: shape factor; FD: Feret's diameter; OM: outer mesocarp; MM: mid mesocarp.

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As illustrated in Figure 2, by moving to the inner part of the pod, the EN was observed to be constituted by two strings of cubic cells of smaller size than those found for the EP and the mesocarp (Figures 2A and 2D); their morphometric parameters were: 640 ± 4.05 μm^2 A, 100 ± 1.34 µm P, 0.80 ± 0.10 SF, and 41 ± 0.81 µm FD; the thickness found by DIA was 0.07 mm. From the inner string of cells of the EN, the TC were observed as simple, long, thin, multicellular structures with markedly observed nuclei. This region showed 0.41 mm thickness. By using the stereomicroscope, the P was observed (Figure 1A); whereas under the light and environmental scanning electron microscopes, the P showed polyhedral cells of different sizes (Figures 2C and 2E). This region ends by forming a thread which was observed under the light microscope, this thread penetrated into the cavity of the pod (locule) where it was 'trapped' by the SE (Figure 2C). Under the stereomicroscope (Figure 1A) the cavity of the vanilla pod showed to be occupied by the SE, which were observed as black, semi-spherical, smooth structures as observed by environmental scanning electron microscopy (Figure 2F). The DIA reported the following information: 61983 ± 40.96 μm^2 A, 994 ± 3.87 µm P, 0.79 ± 0.07 SF, 312 ± 2.69 µm FD.

Spatial Ratio of Identified Compartments

As shown in Figure 3, through the application of DIA to the micrographs of transversal sections of green mature vanilla pods obtained by using the stereomicroscope, five main compartments were identified and the spatial ratio with respect to the whole transversal section in the pseudo-prismatic region of the pods was calculated for each compartment. In Figures 3A to 3E, the segmentation process of images similar to that presented in Figure 1A is shown. Accordingly to the results summarized in Table II, the region composed of EP, OM and MM represents the greatest spatial percentage (66.55%), followed by P, SE, and TC. The intralocular interstitial cell-free region, occupies only 0.04% of the total space. These results are close to those calculated by others authors (*22*); the most remarkable differences can be observed for the values corresponding to the spatial ratio represented by the seeds and the intralocular interstitial cell-free region, 6.20% and 4.70%, respectively as reported in the cited work. The methods

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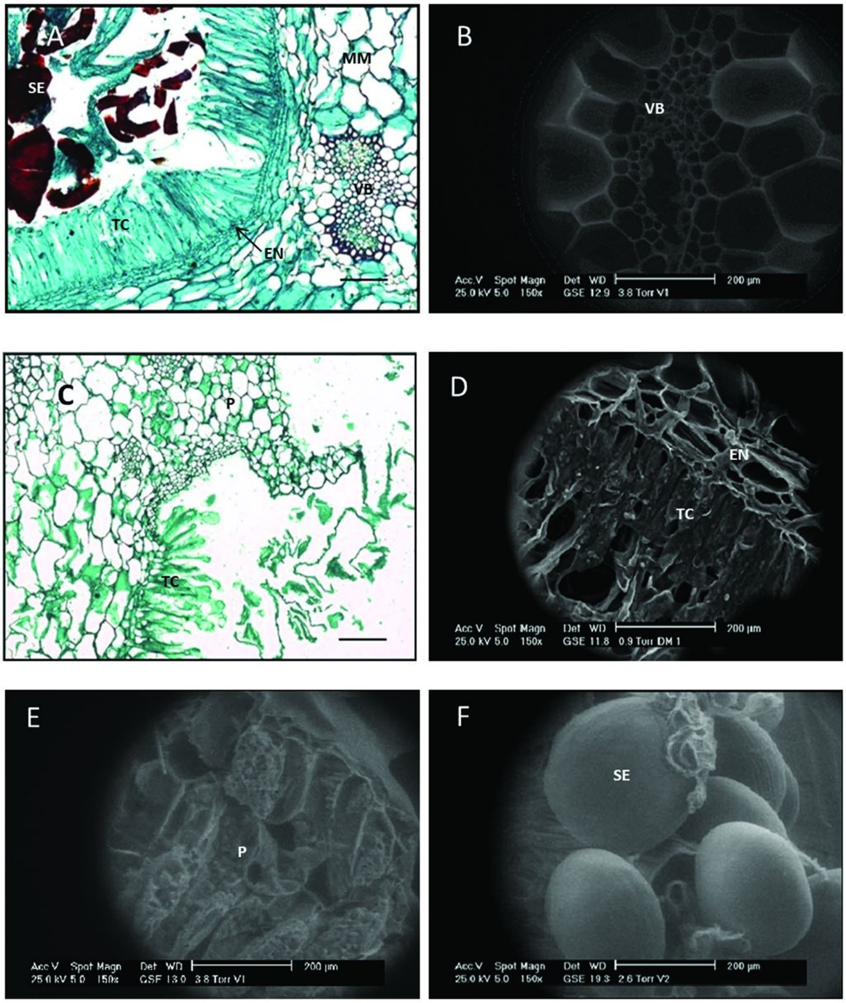
employed by others researchers (*22*) (hand dissection of each region of the frozen green mature pods, and calculation of the relative surface for each compartment taking as a basis the segmentation of an image printed in photographic paper of known weight and density) could be not comparable with the DIA method conducted in the present study.


A
SE
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Acc.V Spot Magn Det WD 200 µm
25.0 kV 5.0 150x GSE 12.9 3.8 Torr V1
C
P
TC
D
EN
TC
Acc.V Spot Magn Det WD 200 µm
25.0 kV 5.0 150x GSE 11.8 0.9 Torr DM 1
E
P
Acc.V Spot Magn Det WD 200 µm
25.0 kV 5.0 150x GSE 13.0 3.8 Torr V1
F
SE
Acc.V Spot Magn Det WD 200 µm
25.0 kV 5.0 150x GSE 19.3 2.6 Torr V2

Figure 2. A – F. Images corresponding to the transversal cut of a green mature pod (capsule) of V. planifolia Andrews; A and C, observations under a light microscope using Safranin O-fast green staining, it is possible to distinguish the vascular bundles (VB) inside the mid mesocarp (MM), as well as the endocarp (EN), trichomes (TC), seeds (SE) and placenta (P); B and D-F, observations with environmental scanning electron microscope: B, vascular bundle (VB); D, detail of endocarp (EN) and trichomes (TC); 11, placenta (P); 12, seeds (SE). The scale value is 200 µm for images A-F.

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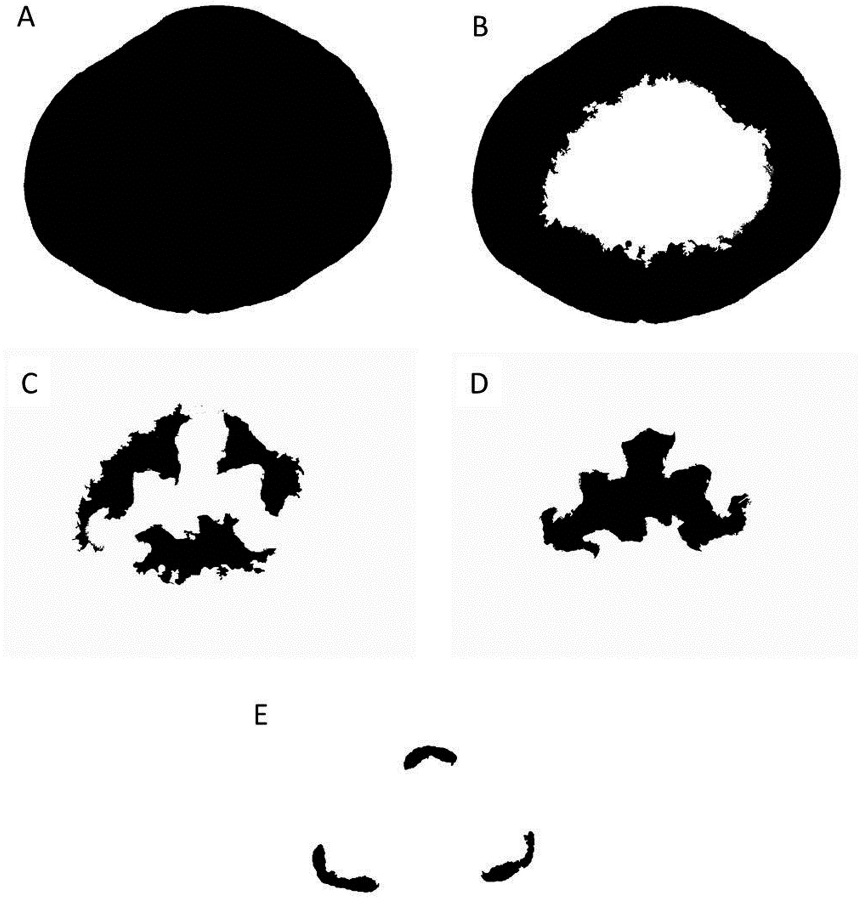

Figure 3. A – E. Segmented, binary images obtained by applying DIA to an image captured with the stereomicroscope, corresponding to the transversal cut of a green mature pod (capsule) of V. planifolia Andrews; in the pseudo-prismatic region; A, whole pod; B, epicarp (EP) + outer mesocarp (OM) + mid mesocarp (MM); C, placenta (P); D, intralocular interstitial cell-free region + seeds (SE); E, trichomes (TC).

Data provided in the present work about microstructure analysis of green mature vanilla pods, among which the discernment of constitutive tissues, cell size and shape, and tissue area are noteworthy, allowed to identify the mesocarp as the main tissue. Previous results (*23*) indicated that structural deformation, mainly at the mesocarp, occurs inside the vanilla pods during the traditional curing process, and this deformation is related to the vanillin concentration. Probably the

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microstructural changes (shrinkage) in the mesocarp during the process allow for more interaction between the active sites of the β-glucosidase and the glycosylation precursors, which are responsible for the synthesis of aromatic compounds in the vanilla pods; this synthesis occurs, as reported by different authors (*9*, *21*), in the tissues adjacent to the mesocarp, such as the placentae and trichomes.

Table II. Spatial ratio of each identified compartment in the pseudo-prismatic region

Compartment	*A[1] (%)*	*B[2] (%)*
Epicarp, outer mesocarp, mid mesocarp and endocarp	64.70	66.55
Placentae	20.30	16.09
Trichomes	4.10	2.90
Seeds	6.20	14.42
Intralocular interstitial cell-free region	4.70	0.04

[1] A: For green mature vanilla (V. planifolia Andrews) pods cultivated in Kerala State (India) and Gutiérrez-Zamora, Veracruz (México) (Data from reference (*22*)). [2] B: The present work.

Conclusions

The structural data obtained by quantifying morphometric parameters through the application of DIA tools to images captured by light microscopy of green mature pods of *V. planifolia* from Papantla de Olarte, Veracruz, Mexico, provided a specific fingerprint of the identity of this particular biological material. This information might be useful to differentiate between distinct vanilla types or species cultivated in different parts of the world, in addition to establish a structural characterization for this specific plant. The use of novel, precise and reliable tools such as DIA constitutes an important contribution to the methodology for acquisition of morphometric data of cells; this allows for comparisons against the results reported by others authors (*22*), on the spatial ratio represented by the intralocular interstitial cell-free region and the seeds inside the vanilla pods cultivated in other parts of the world. Microscopy techniques and digital image analysis were efficient tools for the characterization of microstructure of vanilla pods and the data obtained integrate numerical information that could be useful for traditional curing process.

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Chapter 11

Nutraceutical Changes Induced in Blue and Red Pigmented Maize by Nixtamalization Process

C. Reyes-Moreno, J. Aguayo-Rojas, and J. Milán-Carrillo*

Universidad Autónoma de Sinaloa, Facultad de Ciencias Químico Biológicas,
Programa Regional del Noroeste para el Doctorado en Biotecnología,
Boulevard las Américas, S/N , C.P. 80000, Culiacán, Sinaloa, Mexico
***E-mail: jmilanc@uas.uasnet.mx**

Maize (*Zea mays* L.) is the most domesticated plant in the world. Among the largest diversity of genetic resources of Mexican maize, pigmented genotypes, as purple, red, and blue are the most common. Of the total number of races (landraces) currently existing in Mexico, there are at least 59 that are clearly and consistently distinguishable on the basis of biochemical and morphological characteristics.

Pigmented maize has received increased attention from a nutraceutical perspective owing to its potential health benefits. Phytochemicals such as phenolics, anthocyanins, among others have been previously reported on several genotypes.

The term nixtamalization refers to the alkaline cooking process of converting maize into foods such as tortillas and snack foods (maize chips, tortillas chips, and tacos).

This chapter reviews our current knowledge about the effect of nixtamalization process on the level of total phenolics, anthocyanins and antioxidant activity of nixtamalized products (masa, nixtamal, tortillas, chips) produced from pigmented maize genotypes.

In Hispanic Foods: Chemistry and Bioactive Compounds; Tunick, M., et al.;
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Introduction

Mexico is rich in biological and cultural diversity. There are approximately 22,000 species of flowering plants among approximately 2,500 genera. Over 10% of the genera and 50 to 60% of the species are endemic to Mexico (*1*). Many crop plants used by humans have their origins in Mexico. Hernández (1993) estimated that more than 105 economically important species were utilized by peoples of Mexico prior to the Spanish conquest (*2*). Pre-Columbian peoples of Mexico developed a great number of domesticated plants, the most important being maize (*Zea mays* L.), chile peppers (*Capsicum annuum* L.), beans (*Phaseolus vulgaris* L.) and squash (*Cucurbita* spp.). Maize has played a central role in the agriculture of all indigenous cultures of Mexico (*3*).

Maize (*Zea mays* L.) is the most domesticated plant in the world. Among the largest diversity of genetic resources of Mexican maize, pigmented genotypes, as purple, red, and blue are the most common. Of the total number of races (landraces) currently existing in Mexico, there are at least 59 that are clearly and consistently distinguishable on the basis of biochemical and morphological characteristics (*4*).

Pigmented maize has received increased attention from a nutraceutical perspective owing to their potential health benefits. Phytochemicals such as phenolics, anthocyanins, among others have been previously reported on several genotypes. Although these compounds are considered nonnutritive, interest in antioxidant and bioactive properties has increased due to their potential health benefits. Several studies with pigmented genotypes have shown that they have multiple functional roles such as protection against oxidative stress, antimutagenic activity and colorectal carcinogenesis inhibition (*5–8*).

Maize tortilla is the main basic daily staple food in Mexico and Central America. In the United States, the consumption of tortillas and their products has grown considerably because of the increasing of the popularity of Mexican food and awareness of their health benefits (*9*). Just in 2010 alone, the tortilla industry reached sales of US$ 1.0 trillion in this country (*10*).

The term nixtamalization refers to the alkaline cooking process of converting maize into foods such as tortillas and snack foods (maize chips, tortillas chips, and tacos). Nixtamalization, or lime cooking, is the alkaline cooking of maize kernels in a calcium hydroxide solution. This process is responsible for important physiochemical, nutritional, and sensory characteristics of maize-based products including pericarp removal, calcium incorporation into kernels, improvement in niacin bioavailability, and formation of flavor and color compounds that impart special sensorial characteristics to these products. The lime- cooking also affects the quality of protein, the amount of resistant starch, and concentrations of anthocyanins, vitamins, minerals, and phytic acid (*11*, *12*). However, little is known about the effect of nixtamalization, tortilla baking, and tortilla chip frying on the fate of phytochemicals and antioxidant activity of nixtamalized products, masa, tortillas, and tortilla chips (*13–17*).

In Hispanic Foods: Chemistry and Bioactive Compounds; Tunick, M., et al.;
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This chapter reviews our current knowledge about the effect of nixtamalization process on the level of total phenolics, anthocyanins and antioxidant activity of nixtamalized products (masa, nixtamal, tortillas, chips) produced from pigmented maize genotypes.

Maize Diversity

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Most crop species currently used in modern agriculture were domesticated during a brief period in human history between five and ten thousand years ago. During this process, cultivated plants underwent domestication bottlenecks (*18*) that generally reduced their gene diversity relative to their wild ancestors. Mexico is the center of origin of maize (Zea mays L), which was domesticated about 9,000 years ago from its wild progenitor teosinte (derived from “teocintli” in nahuatl language: “teotl” = sacred and “cintli” = dried ear of maize), a common name give to a group of annual and perennial species of genus (Zea mays ssp. parviglumis) probably in a single event in Central Balsas River Valley of Southwestern Mexico, confirmed by archaeological and molecular evidence (*19–21*). After its domestication in the Central Balsas River watershed, the maize initial spread was in Mexican Central Highlands where its early diversification took place, perhaps favored by an increase in diversity due to the introgression from other teosinte populations (Zea mays spp. mexicana). Once this early diversification occurred, maize dispersal followed two routes, toward North America and South America (*19*).

During the spread of maize cultivation, different maize lineages acquired distinct genetic and morphological characteristics that eventually diversified and adapted to a wide range of climatic and geographic conditions. Sets of plants sharing particular characteristics have been classified into races (landraces). Members of a landrace have pronounced similarities not only in morphological phenotype and geographical distribution, but also in genetic, cytological, physiological, and agronomic characteristics (*22*, *23*). A landrace has been locally sown year after year, and local farmers have dynamically maintained its diversity. With the occurrence of modern agriculture, new varieties have been introduced, often originating from crosses among elite inbred lines. These new varieties as a group usually contain less genetic diversity but also have higher yields; thus, they have replaced old local landraces in some places (*3*).

Among the largest diversity of genetic resources of Mexican maize, pigmented genotypes, as purple, red, and blue are the most common. Of the total number of races (landraces) currently existing in Mexico, there are at least 59 that can be clearly and consistently distinguished on the basis of biochemical and morphological characteristics (*3*, *4*).

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Phytochemicals in Pigmented Maize

Currently, pigmented maize has received an increased attention from a nutraceutical perspective owing to its potential health benefits. Several bioactive constituents, such as phenolic compounds and anthocyanins, among others, have been previously reported on several genotypes; these bioactive compounds are present mainly in whole grains. Although these compounds are considered nonnutritive, interest in antioxidant and bioactive properties has increased due to their potential health benefits (*5–8*). Phenolic acids and flavonoids represent the most common form of phenolic compounds found in whole maize kernel, with a number of types that exist as soluble free and conjugated or insoluble bound forms (*24*). More than 5,000 flavonoids have been identified in nature (*25*). The most significant function of the sap-soluble flavonoids is their ability to impart color to the plant in which they occur. Flavonoids are responsible for most orange, scarlet, crimson, mauve, violet, and blue colors, as well as contributing much to yellow, ivory, and cream colors (*26*). Anthocyanins as a class of flavonoids are water-soluble glycosides of polyhydroxy and polymethoxy derivates of 2-phenylbenzopyrylium or flavylium salts and are responsible for the red, purple, and blue colors of many fruits, vegetables, and cereal kernels (*27*). Simple or acylated anthocyanin pigments are mainly located in the aleurone layer or pericarp of the maize endosperm, greatly affecting the color of the kernel (*28*), and could be separated into anthocyanin-rich fractions for use as functional colorants or functional food ingredients (*29*). They can be found in many different varieties of pigmented maize in Mexico (*30*). They are probably the most important group of visible plant pigments besides chlorophyll. In their natural state, simple anthocyanins are esterified to one or several sugars. When, in addition to this sugar, they also have an acyl radical they are called acylanthocyanins (*31*). Studies conducted by Salinas et al. (2003) indicate that the anthocyanins found in blue maize comes from cyanidin and malvidin (mainly from derivatives of the former), whereas in red grain maize they come from pelargonidin, cyanidin, malvidin and other still undescribed anthocyanins (*32*). Jackman and Smith (1996) found the free and acylated cyanidins, 3-glucosides of pelargonidin and peonidin, in blue maize. In addition to the color that they impart, there is an intensified interest in anthocyanins, as well as other flavonoids and phenolic acids, due to their beneficial health effects (*33*). The health beneficial properties of these plant metabolites have been attributed to their high antioxidant and antiradical activities but also to many other mechanisms such as antimutagenesis, anticarcinogenesis, and estrogenic activities, inhibition of enzymes, and induction of detoxification enzymes (*34*). Anthocyanin rich foods and anthocyanin pigments have been suggested as potential agents to reduce the risk of colon cancer by inhibiting proliferation of human colon cancer cells in vitro (*35*). Also, the tests of Tsuda et al. (2003) provide a nutritional and biochemical basis for the use of the anthocyanins as a “functional food factor” that may be beneficial for helping to prevent diabetes and obesity (*36*).

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Nixtamalization Process

The earliest reported archeological evidence of maize dates back to the wild precursors that existed some 8,000 years ago in Central Mexico. By two thousand years later, maize was already under cultivation and was very much involved in the subsequent rise of the great Mesoamerican civilizations (*37*). Katz et al. (1974) established the hypothesis that all societies who depend on maize as their staple diet also used the technique of alkaline cooking (*38*). For this purpose, they researched 51 societies who lived in America. In basis of this studies concluded that the use of lime is found predominantly in Mesoamerica and only sporadically in southern of United States, which favors the idea that the nixtamalization process was originated between the main societies settled in Mexico and that maize as its grain cooking were introduced later to all the continent (*38*). However, it is not known how and why the Aztec and Mayan civilizations selected this type of process. Although studies of physical, chemical and nutritional change that the nixtamalization process causes in the maize offers several possible explanations. It is very probable that the thermal-alkaline treatment of maize grain had been selected by trial and error; undoubtedly, the explanation that most anthropologists and their informants have offered for the significance of this treatment in each society is that alkali softens and “hulls” the tough pericarp of the maize. Yet, if the alkali cooking techniques used by societies consuming large quantities of maize are examined in the cultural context alone, then they would seem only to be innocuous methods for softening the outer kernel and would carry no adaptive or evolutionary significance. However, the archeological evidence favors the fact that without these cooking techniques a high degree of dependence on maize produces serious malnutrition (*39*).

The process of nixtamalization plays a critical role in the increase of the nutritional value of maize through its utilization in the elaboration of food products such as tortillas, snack tortillas, snack maize and tortillas for tacos (*11*, *40*). The technology for tortilla production is ancestral and it has been transmitted from generation to generation in Mesoamerica. This technology involves the thermal-alkaline cooking of the maize grain, process named nixtamalization (from Nahuatl: *nixtli* = ashes and *tamalli* = cooked dough of maize). In Mexico these techniques are still utilized as in the Aztec times; the nixtamalized maize was ground in a *metate* to produce dough (*masa*) that was used to form disks by hand of approximately 20 cm of diameter, which were cooked on a clay griddle (*comal*) (*11*). The resulting product was called *tlaxcalli* for the Aztecs and subsequently tortilla for the Spaniards (*41*).

The traditional nixtamalization process requires cooking the maize kernels in a solution of water with lime and steeping overnight. Generally, the ratio of maize to solution ranges from one part of whole maize kernels to 3-10 parts of the alkaline solution. The solution is generally a water and lime solution with 2% by weight lime ($Ca(OH)_2$). The maize is cooked by boiling followed by a steep period, on the order of 12 h. This cooking process softens the pericarp and allows the endosperm to absorb water, thus facilitating subsequent milling. After steeping, the solution is drained. The steeped maize is called nixtamal, and the

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cooked-step liquid, rich in maize solids, is called nejayote. Nejayote is a highly alkaline waste product that has a large oxygen demand, and must be disposed properly, which significantly increases the cost of the traditional nixtamalization process. The nixtamalized kernels are then repeatedly washed with water to remove excess lime and any solubilized particles generating additional waste liquid. The cooked and nixtamalized kernels can be ground or milled, in disk mills for example, with the addition of small amounts of water. The resulting dough is called masa and is suitable for making food products, including tortillas and other related tortillas products such as maize chips, tortilla chips, taco shells and nachos (*11*, *40*). The nixtamalization process is responsable for important physiochemical, nutritional and sensory characteristics of maize-based products including the removal of the pericarp, incorporation of calcium into the kernels, improvement of niacin bioavailability, and formation of flavor and color compounds that impart typical sensorial characteristics to the final products (*11*, *12*).

Tortillas are made from the masa by forming thin disks of masa with an appropriate diameter (e.g. 12 to 15 cm). These disks are cooked on both sides to obtain the final tortilla product. Generally, tortillas have a relatively short shelf life in that they quickly harden and lose their flexibility. Traditionally prepared tortillas, without additives, have a maximum shelf life of about 12 to 15 h, and after this time, they become hard or stale. Therefore, discerning tortilla consumers obtain tortillas manufactured the same day. Alternatively, the cooked, nixtamalized and milled kernels can be dried so that the final product will be a nixtamalized maize flour (also called "instant masa" or "dry masa"). This instant masa can be shipped to tortilla manufacture or consumers. Water is added to instant masa to make a reconstituted masa and itis used to make tortillas. However, it is generally believed that the instant masa made from nixtamalized maize flour has lower quality characteristics than fresh masa, as reflected in the final tortilla product flavor and texture.

Maize tortilla is the main basic daily staple food in Mexico and Central America. In the United States, the consumption of tortillas and their products has grown considerably because of the increasing of the popularity of Mexican food and awareness of their health benefits (*9*). Just in 2010 alone, the tortilla industry reached in this country, sales of US$ 1.0 trillion (*10*).

Effect of Nixtamalization on Phenolics, Anthocyanins, and Antioxidant Activity of Pigmented Maize

This nixtamalization process causes profound changes in the structure, chemical composition, and nutritional value of the foods. One of the changes is the partial removal of the pericarp or bran due to alkali treatment, nixtamal washing, and handling (*42*), so that the finished products are considered as semi-whole grain foods. This is important because whole grain consumption has been associated with the prevention of cardiovascular disease (CVD), type

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2 diabetes, and some cancers (*43*, *44*). In addition, the new USDA Dietary Guidelines for Americans emphasizes the need for consumption of whole grains. The phytochemicals present in whole grains are mainly in bound form (*34*, *45*, *46*).

Nutritionally, lime cooking also affects the quality of protein, the amount of resistant starch, and concentrations of anthocyanins, vitamins, minerals, and phytic acid. However, little is known about the effect of nixtamalization, tortilla baking, and tortilla chip frying on the fate of phytochemicals and antioxidant activity of nixtamalized products, masa, tortillas, and tortilla chips (*11*, *13–17*).

The total phenolic content of blue and red raw maize, masa, tortillas, and tortilla chips is presented in Table 1. The range of total phenolic content of raw maize was from 45.1 to 465.3 mg gallic acid equivalents (GAE)/100 g of dry weight of sample. These differences could be attributed to the genetic background, grain physical properties and particularly to the relative ratio of the anatomical parts of the kernel since the pericarp and endosperm's aleurone layer are the structures richest in phenolic compounds (*34*, *47*). The range of total phenolic content for nixtamal or masa and tortillas was from 19.8 to 226.8 and from 19.8 to 240.7 mg GAE /100 g of dry weight of sample (Table 1). There was a significant reduction in phenolic content when corn was nixtamalized or lime-cooked. However, the difference between masa and tortillas indicated that the main losses were incurred during lime-cooking and steeping. In general, these significant losses can be attributed to the combined effect of alkaline and thermal processing during nixtamalization, as well as physical losses of the pericarp and leaching of phenolics into the cooking liquor (nejayote). Nejayote is rich in some important phytochemicals as phenolics compounds, anthocyanins and carotenoids that are leached out during cooking (*48*). Nixtamalization also affects the polyphenolics by breaking down cell wall ester linkages to release free phenolic compounds present in the pericarp into the cooking solution (*49*) and is responsible for partial breakdown of the kernels.

Del Pozo-Insfran et al. (2006) and Lopez-Martínez et al. (2011) reported a reduction between 51.7 and 75.0% in total phenolic content assayed in tortillas of pigmented blue (Mexican and USA) and red (Mexican) maize genotypes (Table 1) (*13*, *17*). Other researchers (*16*) observed high retention of phenolic compounds (60.9-63.9%) in pigmented maize tortillas prepared with instant nixtamalized maize flours from blue and red maize (Table 1). The highest concentration and stability of the phenolics in the last tortillas might be mainly attributed to the application of optimal nixtamalization conditions for kernels processing.

Mora-Rochin et al. (2010) observed that most phenolics (>80%) in raw kernels pigmented maize and their nixtamalized products occurred in the bound or attached to cell wall form (*16*). Similar findings were reported by other researchers (*15*, *46*) who indicated than 80% of total phenolic content in maize and others cereal grains are bound primarily to hemicellulose in cell wall of the pericarp, aleurone layer and germ.

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Table 1. Total phenolic content of raw pigmented maize and their nixtamalized products

			Total phenolics (mg gallic acid/100, dw)						
			Free		*Bound*		*Total*		
Maize/type	*n*	*Products*					*Total*	*%*	*References*
Blue (Mex)	1	Raw					45.1		*Del Pozo-Insfran et al (2006)*
Blue (Mex)	1	Nixtamal					19.8	↓54.0	
Blue (Mex)	1	Tortilla					19.8	↓56.1	
Blue (Mex)	1	Chips					12.0	↓73.4	
Blue (USA)	1	Raw					131.0		
Blue (USA)	1	Nixtamal					29.8	↓66.0	
Blue (USA)	1	Tortilla					29.8	↓75.0	
Blue (USA)	1	Chips					20.0	↓81.0	
Blue (Mex)	1	Raw	410						*Del Pozo-Insfran et al (2007)*
Blue (Mex)	1	Tortilla	172	↓58.0					
Blue (Mex)	1	Chips	151	↓63.2					
Blue (Mex)	1	Raw	1,210						
Blue (USA)	1	Tortilla	278	↓77.0					
Blue (USA)	1	Chips	217	↓82.1					
Red (USA)	1	Raw	38		206		243.8		*De la Parra et al (2007)*
Red (USA)	1	Masa	28	↓26.7	97	↓52.7	125.3	↓48.6	
Red (USA)	1	Tortilla	31	↓20.2	106	↓48.4	136.5	↓44.0	
Red (USA)	1	Chips	26	↓30.9	85	↓58.5	111.7	↓54.2	
Blue (USA)	1	Raw	46		221		266.2		
Blue (USA)	1	Masa	30	↓33.4	128	↓41.9	158.5	↓40.5	
Blue (USA)	1	Tortilla	39	↓14.1	123	↓44.4	161.8	↓39.2	
Blue (USA)	1	Chips	41	↓9.0	96	↓56.7	136.9	↓48.6	

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Table 1. (Continued). Total phenolic content of raw pigmented maize and their nixtamalized products

Maize/type	*n*	*Products*	*Total phenolics (mg gallic acid/100, dw)*						*References*
			Free		*Bound*		*Total*		
							Total	*%*	
Blue (Mex)	1	Raw	27		115		142.1		Mora-Rochín et al 2010
Blue (Mex)	1	Tortilla	17	↓36.3	69	↓39.7	86.6	↓39.0	
Red (Mex)	1	Raw	27		113		140.7		
Red (Mex)	1	Tortilla	17	↓37.7	73	↓35.7	90.0	↓36.0	
Blue (Mex)	1	Raw					343.2		***López-Martínez et al (2011)***
Blue (Mex)	1	Masa					195.4	↓56.9	
Blue (Mex)	1	Tortilla					201.4	↓58.7	
Red (Mex)	1	Raw					465.3		
Red (Mex)	1	Masa					226.8	↓48.7	
Red (Mex)	1	Tortilla					240.7	↓51.7	

The anthocyanin content of blue and red maize and their nixtamalized products is presented in Table 2. Anthocyanins are the main compounds responsible for the final tortilla color. These antioxidant compounds are mainly found in the pericarp and/or aleurone layer (*50*). In general, most anthocyanins were lost during lime-cooking by leaching into the steep solution or nejayote. There were significant differences in anthocyanin content among types of maize; red maize contained the highest amount (779 mg of cyanidin-3-glucoside equiv/100 g, dry weight basis), followed by blue maize (192 mg of cyanidin-3-glucoside equiv/100 g, dry weight basis). Lime-cooked blue and red maize kernels lost approximately 38.26-92.86% and 48.72-100% of the anthocyanins present in their respective raw kernels (Table 2). These significant losses occurred as a result of the synergistic effect of the alkaline pH and temperature, which causes important structure transformations or modifications in the anthocyanins. In this regard, Jing & Giusti (2007) concluded that maize anthocyanins are complexed and precipitated with denatured proteins after cooking (~100°C), leading to a decline in total assayable anthocyanins (*51*). Also, studies conducted by Fossen et al. (1988) indicate that cyanidin 3-glucoside losses stability when the pH is higher than 5 as always occurs during nixtamalization (pH > 6.5) (*52*). Differences in anthocyanin content among nixtamal or masa, tortilla and tortillas chips were observed; the highest losses (60.5-91.07%) were observed in tortilla chips (Table 2).

Table 2. Anthocyanin content of raw pigmented maize and their nixtamalized products

Maize/Type	***n***	***Products***	***Anthocyanin content*** *(mg of cyanidin-3-glucoside equiv/100 g, dw)*		***References***
				%	
Blue (Mex)	8	**Raw**	115 to 192		***Salinas-Moreno et al (2003)***
Blue (Mex)	8	NMF	71 to 117	↓38.26-39.06	
Red (Mex)	5	**Raw**	35 to 779		***Salinas-Moreno et al (2003)***
Red (Mex)	5	NMF	0 to 87	↓88.83-100	
Blue (Mex)	1	Raw	10.07		***Agama-Acevedo et al (2004)***
Blue (Mex)	1	Nixtamal	3.09	↓69.31	
Blue (Mex)	1	Raw	27.18		***Cortés et al (2006)***
Blue (Mex)	1	NMF	4.936	↓81.83	
Blue (Mex)	1	Raw	32.10		***Del Pozo-Insfran et al (2006)***
Blue (Mex)	1	Nixtamal	17.40	↓45.79	
Blue (Mex)	1	Tortilla	14.80	↓53.89	
Blue (Mex)	1	Chips	7.60	↓76.32	
Blue (USA)	1	Raw	30.70		***Del Pozo-Insfran et al (2006)***
Blue (USA)	1	Nixtamal	16.00	↓47.88	
Blue (USA)	1	Tortilla	11.50	↓62.54	
Blue (USA)	1	Chips	5.80	↓81.10	
Blue (Mex)	1	Raw	34.22		**Del Pozo-Insfran et al (2007)**
Blue (Mex)	1	Tortilla	15.40	↓55.00	
Blue (Mex)	1	Chips	13.52	↓60.50	

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Table 2. (Continued). Anthocyanin content of raw pigmented maize and their nixtamalized products

Maize/Type	*n*	*Prod-ucts*	***Anthocyanin content*** *(mg of cyanidin-3-glucoside equiv/100 g, dw)*	*%*	*References*
Blue (USA)	1	Raw	26.09		***Del Pozo-Insfran et al (2007)***
Blue (USA)	1	Tortilla	7.04	↓73.00	
Blue (USA)	1	Chips	5.22	↓80.00	
Blue (USA)	1	Raw	36.87		***De la Parra et al (2007)***
Blue (USA)	1	Masa	2.63	↓92.86	
Blue (USA)	1	Tortilla	3.81	↓89.66	
Blue (USA)	1	Chips	3.29	↓91.07	
Blue (Mex)	1	Raw	30.69		***Mora-Rochín et al 2010***
Blue (Mex)	1	Tortilla	13.80	↓55.03	
Blue (Mex)	1	Raw	63.10		***Lopez-Martinez et al (2011)***
Blue (Mex)	1	Masa	27.90	↓55.78	
Blue (Mex)	1	Tortilla	18.90	↓70.04	
Red (Mex)	1	Raw	82.30		***Lopez-Martinez et al (2011)***
Red (Mex)	1	Masa	42.20	↓48.72	
Red (Mex)	1	Tortilla	33.50	↓59.29	

Table 3 shows the hydrophilic antioxidant activities, or ORAC values, of free and bound phenolics of the raw blue and red maize and their nixtamalized products as micromoles Trolox equivalents per 100 g of sample, respectively. The range of antioxidant activity for blue and red raw grains was from 2,560 to 12,286 and 2,115 to 19,190 μmol Trolox equivalents (TE)/100 g of dry weight of sample, respectively. It was observed that bound phenolics were the primary contributors (64.7 to 74.5%) of antioxidant activity. Some researchers (*15*, *17*, *34*) have reported that total polyphenolics are the most important contributors (98%) to the total antioxidant capacity in maize. In general, most researchers (*13*, *14*, *16*) have reported that the total antioxidant activity decreased after lime-cooking process; tortillas and tortilla chips from nixtamalized pigmented maize types lost 16.34 to 51.03 % and 58.69 to 61.48 %, respectively, of the ORAC value observed in raw kernels (Table 3). However, Gutiérrez-Uribe et al. (2010) observed that the free and bound antioxidant activities of masa solids were approximately 4.8 and 5 times higher compared to raw kernels (Table 3) (*53*); according to these researchers their results clearly indicate that lime-cooking enhanced free and bound antioxidant activities. These results are not comparable to other previous reports that only considered the antioxidant activity of free compounds extracted in phosphate buffer solution and that indicate that less than 80% of the antioxidant activity of maize was recovered in masa (*14*). In addition, the short lime-cooking times applied by Gutiérrez-Uribe et al. (2010) to the kernels under study did not allow enough time to release or degrade bound phytochemicals (*53*).

Table 3. Antioxidant activity of raw pigmented maize and their nixtamalized products

			Antioxidant activity^1					
Maize/Type	***n***	***Products***	***Free***	***Free %***	***Bound***	***Bound %***	***Total***	***References***
Blue (Mex)	1	Raw					2,900	***Del pozo-Insfran et al (2006)***
Blue (Mex)	1	Nixtamal					1,520	
Blue (Mex)	1	Tortilla					1,420	
Blue (Mex)	1	Chips					1,198	
Blue (USA)	1	Raw					2,560	
Blue (USA)	1	Nixtamal					1,498	
Blue (USA)	1	Tortilla					1,320	
Blue (USA)	1	Chips					1,000	
Blue (Mex)	1	Raw					2,960	
Blue (Mex)	1	Tortilla					2,116	***Del pozo-Insfran et al (2007)***
Blue (Mex)	1	Chips					1,184	
Blue (USA)	1	Raw					2,560	
Blue (USA)	1	Tortilla					1,792	
Blue (USA)	1	Chips					986	
Blue (Mex)	1	Raw	3,880		8,406		12,286	
Blue (Mex)	1	Tortilla	1,719	↓55.7	8,555	↑1.8	10,274	***Mora-Rochín et al (2010)***
Red (Mex)	1	Raw	4,159		15,032		19,190	
Red (Mex)	1	Tortilla	2,422	↓41.8	9,088	↓39.5	11,510	

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Table 3. (Continued). Antioxidant activity of raw pigmented maize and their nixtamalized products

			Antioxidant activity[1]					
Maize/Type	***n***	***Products***	***Free***	***Free %***	***Bound***	***Bound %***	***Total***	***References***
Red (Mex)	1	Raw	35		1,765		2,115	
Red (Mex)	1	Masa	167	↑377.4	5,660	↑220.7	7,331	***Gutiérrez-Uribe et al 2010***
Blue (Mex)	1	Raw	33		1,688		2,021	
Blue (Mex)	1	Masa	165	↑396.4	5,860	↑247.1	7,513	

[1] μmol Trolox equivalents (TE)/100 g of dry weight of sample.

Conclusions

Nixtamalized products (nixtamal or masa, tortillas, tortilla chips) of blue and red pigmented maize processed by traditional nixtamalization process contained and retained phenolics, anthocyanins and antioxidant activity differently. Nixtamalization process promoted the loss of phenolics and antioxidants but the use of optimal processing conditions, with short lime-cooking times, generated significantly lowest losses or increases in this parameters. However, more research is needed to find the best processing conditions to retain or increase the phytochemical content and functional properties of pigmented maize in nixtamalized food products, which is of interest due to the increasing consumer preference for maize-based products and for their possible contribution to the human diet owing to the antioxidant activity and potential nutraceutical properties.

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Chapter 12

In Vitro Evaluation of the Antidiabetic and Antiadipogenic Potential of Amaranth Protein Hydrolysates

Aída Jimena Velarde-Salcedo,[1] Elvira González de Mejía,[2] and Ana Paulina Barba de la Rosa*,[1]

[1]Instituto Potosino de Investigación Científica y Tecnológica A. C., Camino a la presa San José 2055, San Luis Potosí, SLP 78216, México
[2]Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, 228 E.R. Madigan Lab, MC-051, 1201 W. Gregory Drive, Urbana, IL 61801
***E-mail: apbarba@ipicyt.edu.mx**

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Obesity, which is a major recognized risk factor for type-2 diabetes, is rapidly increasing in prevalence resulting in a "diabesity" epidemic. Diabesity represents one of the major public health problems in the 21st century. Some of the strategies that have shown to be effective in reducing type-2 diabetes incidence are exercise and a healthy diet. New drugs that have as a target the inhibition of the enzyme dipeptidyl dipeptidase IV (DPPIV) have been released. However some of these drugs have secondary effects; for that reason, the food industry is exploring the aspects related to the components present in food that promote a healthy life, such as the bioactive peptides encrypted in the proteins of several foods. Amaranth is a plant native from North, Central and South America which contains antihypertensive, antioxidant and cancer preventive peptides. Also there is evidence that amaranth has some hypoglycemic action; however, the antidiabetic potential and the effect upon body weight of the seed proteins have not been well characterized. The aim of this study was to identify the ability of amaranth peptides to inhibit the DPPIV activity and the effect of these peptides upon fat accumulation in mouse adipocyte cultures.

In Hispanic Foods: Chemistry and Bioactive Compounds; Tunick, M., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

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Introduction

The modern food industry is exploring the aspects related to the components present in food that promote the well-being and a healthy life in the consumer (*1*). It has been reported that food proteins and peptides, aside from being basic macronutrients, also present a wide variety of biological activities that could have a beneficial effect in human health acting as antioxidants, antihypertensives, antithrombotics, among others (*2*). Biologically active peptides are small sequences of amino acids encrypted in food proteins that are activated when released by proteolytic enzymes, during food processing or during intestinal digestion (*3*). Milk and its derivatives are one of the richest sources of biopeptides, but biopeptides are also found in other animal and vegetables sources like meat, eggs, fish, wheat, corn, soybean, rice, mushrooms and amaranth (*2–5*).

Amaranth Nutraceutical Properties

Amaranth is a plant native from North, Central and South America cultivated since pre-Columbian times and in the 1980’s was declared a crop with a high biotechnological potential (NAS 1984). In the last decade amaranth has emerged not only for its nutritional value, but also for its nutraceutical properties (*6*). The presence of several compounds in amaranth seeds like phytosterols, flavonoids, essential oils and protein extracts have been reported. This evidence explains the use of amaranth as an hypocholesterolemic, antioxidant and hypoglucemic plant (*7–11*).

In our research group we have identified the presence of peptides with different biological properties (*5*) encrypted in amaranth proteins, which include peptides with an inhibitory activity upon the angiotensin-converting enzyme (ACE) that has an antihypertensive effect. These peptides have been characterized using the coronary endothelial cell line and aortic rat rings showing the vasorelaxing effect via nitric oxide production ((*12*). Fritz et al. (*13*) have also demonstrated the blood pressure lowering effect of amaranth hydrolysates in hypertensive and normotensive rats.

It has also been reported that the lunasin-like peptide is present in amaranth proteins (*14*). Lunasin is a novel biopeptide reported for the first time in soybean that is capable of inhibiting cancer development (*15*). Both soybean and amaranth lunasin contains the domains that are required for the internalization of the protein and for its binding to chromatin in cancer cells. It was determined that amaranth lunasin-like peptide internalize into the cell and reaches the nucleus more rapidly than soybean lunasin, where it prevents cell malignization in the presence of a carcinogenic agent and also inhibits H_3 and H_4 histone acetylation. This evidence shows the anticancer properties of amaranth seeds (*14*).

Amaranth and Type-2 Diabetes

The dipeptidyl peptidase IV (DPPIV) is responsible for the degradation of the major insulinotropic hormones, the incretins. Incretins are peptidic hormones released to the blood stream after food intake by the enteroendocrine cells of the small intestine (Figure 1). These hormones immediately stimulate the synthesis and excretion of insulin by pancreatic β cells and therefore decrease the glucose blood levels (*16–18*). However, the lifetime of incretins is very short because they are rapidly inactivated by DPPIV. Hence, the use of DPPIV inhibitors increases the time of action of these hormones and potentiate their effects (*19–21*). There are several DPPIV inhibitors available that have shown promising results as antidiabetic agents (*22–24*), and the search of natural products with a similar activity is always a viable alternative for metabolic diseases and opens up a new investigation field.

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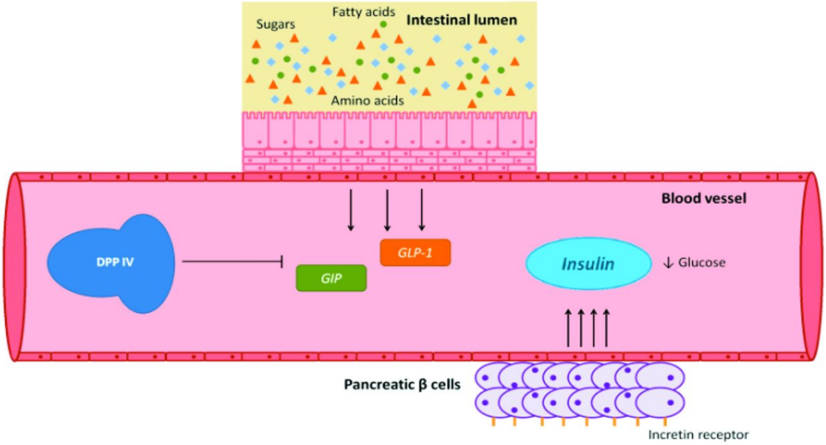

Figure 1. Schematic representation of incretin secretion and action. GIP and GLP-1 are secreted after food ingestion, and they then stimulate glucose-dependent insulin secretion. Once released, incretins are inactivated by DPPIV.

Using mass spectrometry data and the biopep database (http://www.uwm.edu.pl/biochemia/index.php/en/biopep), it was found that in addition to the hypertensive and cancer-preventive peptides reported in amaranth glutelins (*5*), this fraction also contained peptides related to the inhibitory activity of the DPPIV. Then the activities of these peptides were tested *in vitro*.

Glutelins were digested with trypsin, using different enzyme:substrate ratios to obtain different degrees of hydrolysis. As shown in Figure 2A, it was found that the intact glutelins had no significant effect upon DPPIV activity, but when they were digested with trypsin the DPPIV activity decreased in a dose-dependent manner. This inhibitory activity was higher when the degree of hydrolysis increased, reaching up to 80% of inhibition of the enzyme (IC_{50}= 1.2 mg/ml).

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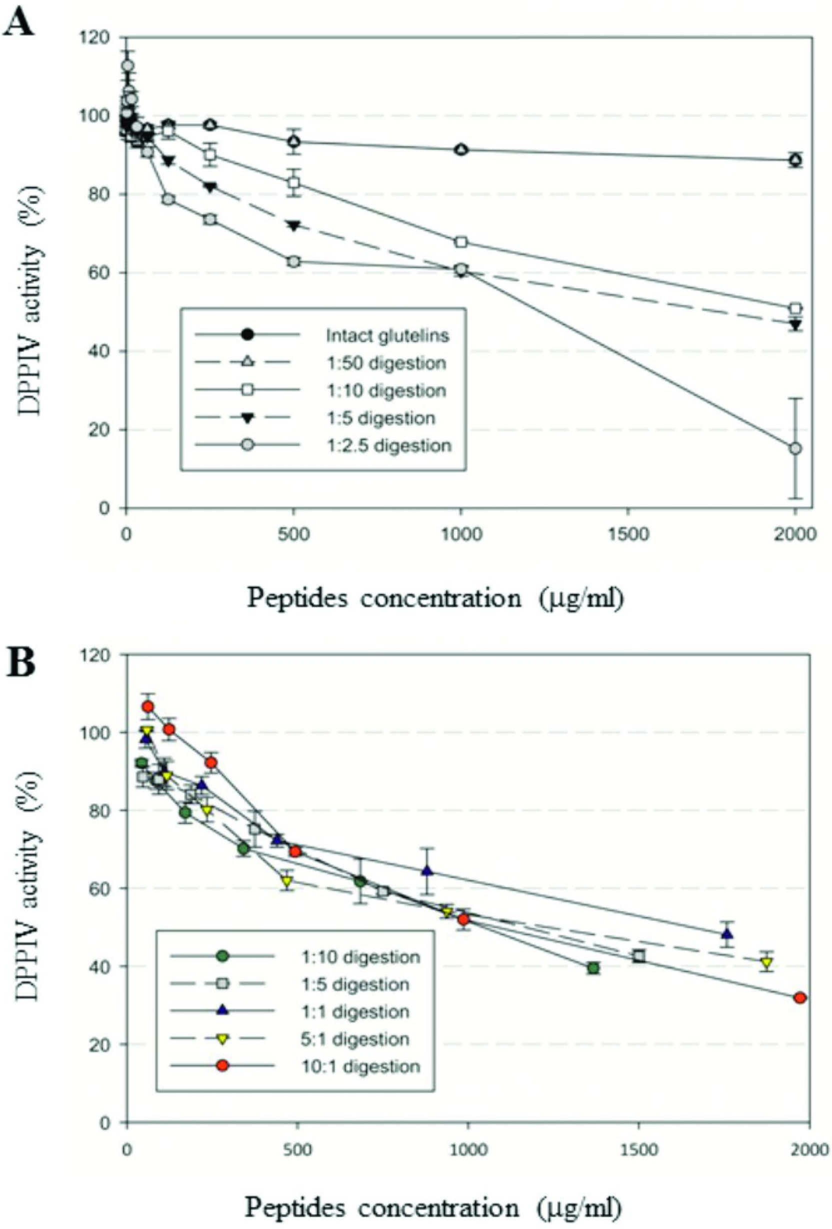

Figure 2. DPPIV inhibitory activity of tryptic glutelins hydrolysates. (A) Amaranth glutelins were digested with trypsin using different enzyme:substrate ratios; (B) fragments bigger than 10 kDa were removed by ultracentrifugation and DPPIV inhibitory activity was measured.

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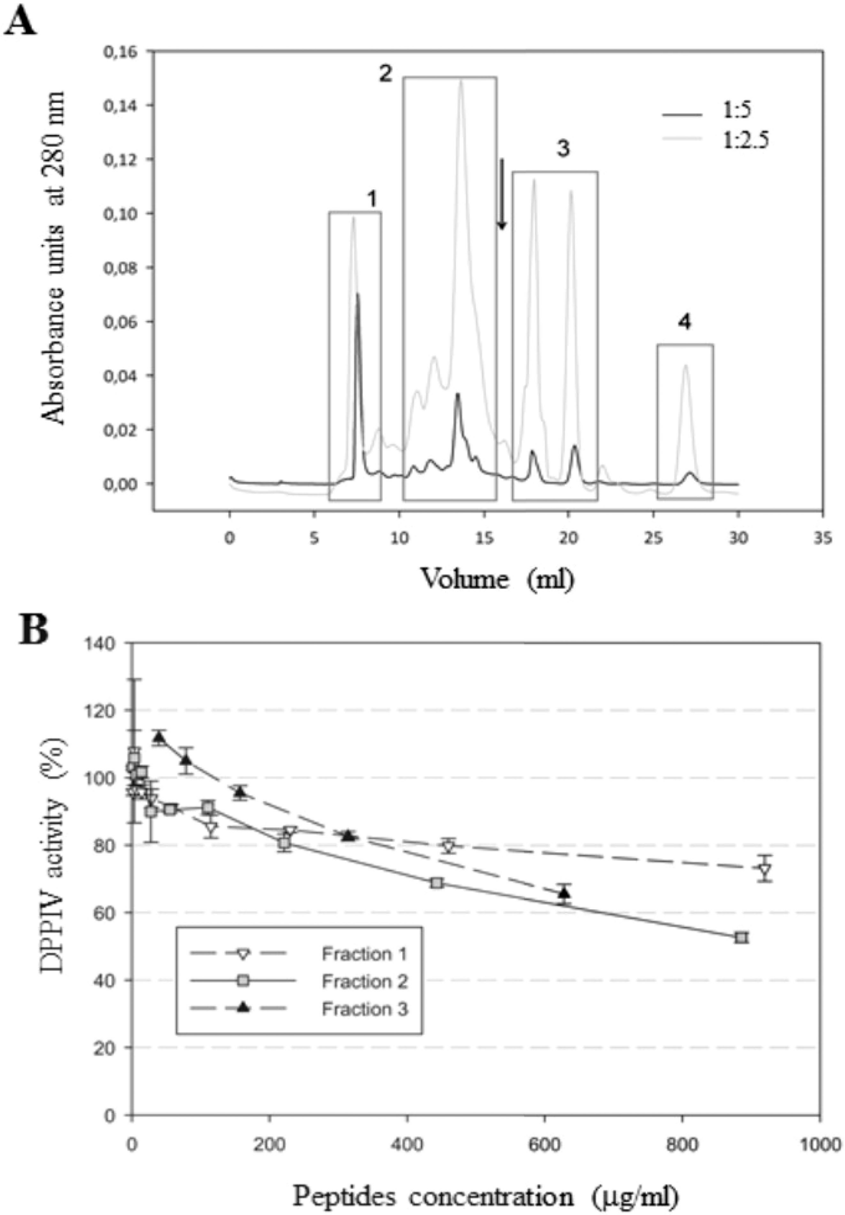

Figure 3. Isolation of DPPIV inhibitory peptides from amaranth gluelins. (A) Tryptic glutelins hydrolysates were separated by size exclusion chromatography. Boxes indicate the four (1-4) fractions collected. Arrow indicates the elution volume of a known DPPIV inhibitor, a tripeptide, diprotin A, as a reference. (B) The main peaks were collected, pooled and tested for their inhibitory activity upon DPPIV.

Interestingly, when the bigger fragments of the hydrolysates were removed by ultrafiltration using a 10 kDa MWCO, it was observed that all the hydrolysates had the same activity, with no significant differences among them (Figure 2B) with IC_{50}s between 1.1 and 1.2 mg/ml. This could indicate that big peptides (larger than three amino acids) can also inhibit DPP IV.

The glutelins hydrolysates obtained at 1:5 and 1:2.5 enzyme:substrate ratio were fractionated by size exclusion chromatography using a column with a separation range between 100 and 7000 Da (Figure 3A). The same separation profile was obtained with both digestions, however as the amount of enzyme increased, the amount of released peptides also increased (peak area). The peaks were collected and pooled by freeze-drying in four fractions, peaks 3 and 4 were collected in the same fraction. Fractions 1, 2 and 3 showed the inhibitory activity upon DPPIV (Figure 3B), where the fraction 1 had the lowest values and both fraction 2 and 3 presented a very similar pattern of inhibition. No activity was found in fraction 4, perhaps because this fraction contained free amino acids, based on the elution volume of the tripeptide (shown with an arrow in Figure 3A). With this information, it was hypothesized that not only small peptides are able to inhibit DPPIV, but apparently bigger fragments can also be considered as potential inhibitors. Docking modeling strategies are useful to predict the molecular mechnisms of action and further work is being carried out.

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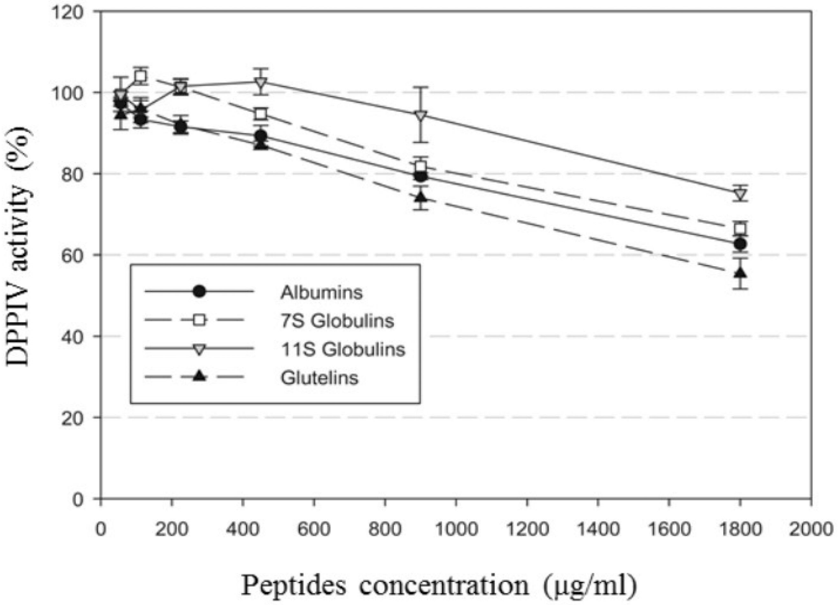

Figure 4. DPP IV inhibitory activity of amaranth seed storage proteins. Tryptic hydrolysates from amaranth seed storage proteins were prepared and tested on the DPP IV activity assay.

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Tryptic digestions of other amaranth storage proteins fractions were tested. Albumins and globulins also had an inhibitory activity upon DPPIV, but 11S globulins had the lowest value (Figure 4).

The gastrointestinal digestion model using different enzymes present during regular digestion was established in order to determine if amaranth consumption could also release these inhibitory peptides. The enzymes used were pepsin released in the stomach, trypsin and pancreatin, which includes lipase, nucleases and other proteases. With this method, the glutelins hydrolysates showed a pattern similar to tryptic hydrolysates (Figure 5). Flours obtained from two amaranth species, *A. hypochondriacus* and *A. cruentus* were compared as well as the flour from popped amaranth. No differences were found among species (IC_{50}= 1.1 mg/ml for *A. hypochondriacus* and 1.4 mg/ml for *A. cruentus*) but the inhibitory activity of flour from popped amaranth decreased considerably compared with the raw flours. This indicates that the heating process is affecting the hydrolysis and availability of bioactive peptides, maybe due to the denaturalization of the proteins, hydrolysis, cross-linking, peptide fragmentation or Maillard reactions between carbohydrates and proteins (*25*). Commercial amaranth supplement was also tested showing that this sample had the lowest inhibitory activity of all amaranth samples. Other seeds were tested. Black bean, which barely had an inhibitory activity upon DPPIV; soybean, which had a higher inhibitory activity than black bean but not as much as amaranth; and wheat, which presented the same pattern as the raw amaranth flours (IC_{50}= 0.8 mg/ml). It seems that the effects upon DPPIV are shared among cereals or seeds with a similar protein distribution. Further work needs to be done.

Amaranth and Obesity

It has been reported that amaranth proteins have antilipemic and weight-lowering effects in rats, indicating a possible potential of amaranth against obesity (*26–28*). The 3T3-L1 cell line of mouse fibroblasts that can be differentiated into adipocytes using specific hormones is widely used as a model of study. The cells were cultured in presence and absence of the tryptic glutelins hydrolysates throughout and after the differentiation process and lipid accumulation using oil red O staining was measured.

Cells cultured in control conditions accumulated a considerable amount of fat, whereas the treated cells produced a lower amount (Figure 6). It was observed that when the cells were treated with the hydrolysates during the whole differentiation process, the final lipids generated decreased significantly, up to a 50% using 100 µg of hydrolysate. This effect seems to be more potent in the cells that are already differentiated where a higher effect was observed. This may indicate that the effect relies more in the lipid synthesis than in the whole signaling pathways involved in differentiation. However, these are preliminary results and more refined experiments need to be done.

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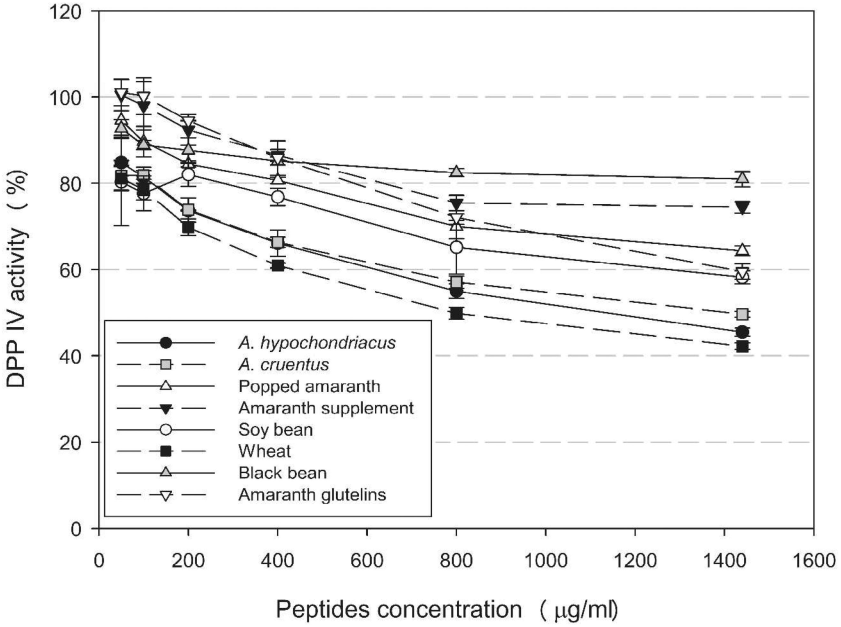

Figure 5. DPPIV inhibitory activity of different seeds sources. A gastrointestinal digestion model was performed on the defatted flour of several amaranth products and other common seeds. The released peptides after digestion were used in the DPPIV activity assay.

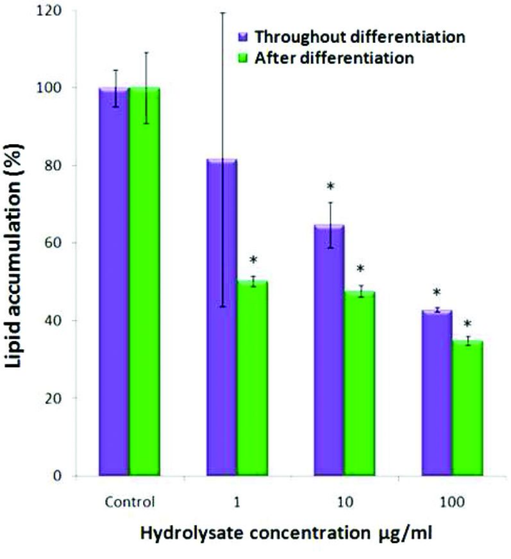

*Figure 6. Anti-lipogenic effect of amaranth tryptic glutelins hydrolysates upon 3T3-L1 cell line. *p<0.001.*

Conclusions

It was shown the presence of DPPIV inhibits peptides encrypted in amaranth seed proteins and its antilipogenic action. These peptides should be released during the digestion process. Amaranth could represent a natural source of these peptides and there should be a lower concern about adverse effects, giving a newer perspective of cereal benefits beyond its nutritional properties.

Acknowledgments

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Chapter 13

High-Antioxidant Capacity Beverages Based on Extruded and Roasted Amaranth (*Amaranthus hypochondriacus*) Flour

Jorge Milán-Carrillo, Alvaro Montoya-Rodríguez, and Cuauhtémoc Reyes-Moreno[*]

Programa Regional del Noroeste para el Doctorado en Biotecnología, Universidad Autónoma de Sinaloa, Av. de las Américas y Josefa Ortíz de Domínguez, Ciudad Universitaria, Culiacán, Sinaloa, 80030, Mexico
[*]E-mail: creyes@uas.uasnet.mx

The objectives of this research were: (1) to determine the best combination of roasting process variables for the production of a high antioxidant roasted amaranth flour (RAF) suitable to elaborate a nutraceutical beverage, (2) to produce high antioxidant extruded amaranth flour (EAF) suitable to elaborate a nutraceutical beverage using extrusion process conditions previously optimized, and (3) to produce two nutraceutical beverages from RAF and EAF. Roaster operation conditions were obtained from a factorial combination of roasting temperature (RT, 110-160°C) and roasting time (1-10 min). Response surface methodology was employed as an optimization technique; the numeric method was applied to obtain maximum values for antioxidant capacity (AoxC) and water solubility index (WSI). The best combination of roasting process was: RT=127°C / Rt= 5.72 min. The best combination of extrusion process variables, obtained in previous studies, was: Extrusion temperature (ET) = 127°C / Screw speed (SS) = 130 rpm. The optimized RAF and EAF had an antioxidant activity of 7,053 and 5,046 μmol Trolox equivalents/ 100 g sample (dw), respectively. A 200 mL portion of the beverages prepared with 22 g of RAF or EAF contained 3.01-3.13 g proteins, 1.22-1.35 g lipids, 14.1-14.3 g carbohydrates and 80 Cal. This portion covers 23.1-24.9 % and 15.5-16.7% of the

In Hispanic Foods: Chemistry and Bioactive Compounds; Tunick, M., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

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daily protein requirements for children 1-3 and 4-8 years old, respectively. A 200 mL portion of the beverages from RAF and EAF contributes with 28-47% and 20-33% of the recommended daily intake for antioxidants, respectively. Both beverages were evaluated with an average acceptability of 8.1-8.4 (level of satisfaction between “I like it” and “I like it extremely”). The high nutritional, antioxidant and sensory value of the beverages can be attributed at least partially to the application of the optimum roasting and extrusion processing conditions. The nutraceutical beverages could be used for health promotion and disease prevention as an alternative to beverages with low nutritional / nutraceutical value.

Introduction

Amaranth is a pseudo-cereal that has been widely grown by the Aztecs, Incas and Mayas in Latin America since pre-Columbian times for millennia; this grain belongs to dicotyledonous class in *Amaranthaceae* family. Interest in its widespread consumption for human nutrition has grown in the two last decades due to favorable reports of amaranth nutritive value and health benefits. Amaranth grains have an excellent nutritional quality; they contain approximately 15% protein with an adequate balance of aminoacids, high lysine content, 60% starch and 8% fat (*1*). The amaranth protein has a high concentration of essential amino acids, especially lysine which is a limited amino acid in other crops; amaranth protein quality is very high and it has a performance comparable to the cheese when amaranth is studied in humans (*2*). The amaranth has higher concentration of soluble fiber than others cereals, such as wheat, corn or oats. Its lipid composition presents polyunsaturated acids fats and squalene (*3*). Moreover, amaranth has many minerals, such as calcium, sodium, iron, magnesium and vitamin E (*4*). Polyphenolic compounds, such as phenolic acids and flavonoids, have been characterized in amaranth grains (*5*). In addition to its promising nutritional qualities, amaranth grains are considered to be an important source of food for celiac patients, since they are gluten-free (*6*), diabetic (*7*), hypercholesterolemic subjects (*8*, *9*) and coronary heart disease and hypertension patients (*10*).

In Mexico, amaranth is cultivated in small amounts, but in the last years the production has been increased, because of, largely, their nutritional and agronomical features (*11*). The national production of this grain in 2008 was 3,864 tons, while in 2009; the production had an increment, reaching the amount of 4,494 tons (*12*). Commonly, amaranth is consumed popped or roasted.

To increase the nutritional value of amaranth, grain is treated by popping and roasting at 170 to 190°C. Heat processing can cause damage of essential amino acids resulting in decreased contents or transfer into a racemic mixture; temperatures above 100°C induce reactions in both bound and free amino acids, and especially in essential amino acids valine, leucine, isoleucine, lysine, threonine, methionine, phenylalanine and tryptophan, which then become

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non-utilizable for humans, thus decreasing the biological value of foodstuffs (*13*, *14*). An important interfering factor on both technological and nutritional qualities of popped or roasted products is the moisture of the feeding material (*9*). The amaranth processed under conditions that do not damage its protein and its essential amino acids availability, like moist heat cooking and extrusion, presents good protein quality, similar to casein (*15*).

Extrusion is a high temperature / short time technology that offer numerous advantages including versatility, high productivity, low operating costs, energy efficiency, high quality of resulting products and an improvement in digestibility and biological value of proteins (*16*). The versatility of the extrusion process has allowed its use to elaborate several food products, including breakfast cereals, snacks, and precooked flours. The use of extruded flours to elaborate some food products has several advantages, since the extrusion process is accompanied by pre-gelatinization of starch granules, resulting in loss of the molecular order and the complete degradation of polymers with the formation of highly soluble fragments. Therefore, suspensions of flours precooked by extrusion are able to increase their viscosity rapidly, with a low tendency to form lumps, since starch granules have been modified showing high swelling capacity under both cold and hot conditions, which makes extruded flours highly recommended for preparation of instant food products (*17*) such as beverages.

In Mexico, instant flours from nixtamalized maize, as well as flours from raw, roasted, germinated, and fermented maize are used to elaborate beverages traditionally consumed as atole, pinole, tesgüino and pozol (*18*). Also, roasted amaranth flour has been employed for preparing a nutritional amaranth drink (amarantole) (*19*).

Nutraceutical beverages represent one of the fastest annual growing markets worldwide, reaching a compound annual growth rate of 13.6 % between 2002 and 2007 (*20*) The main criterion for acceptance of this kind of drinks is the taste and acceptability; thus, formulation of high quality beverages with good taste is important for their adequate levels of consumption, which are needed for health promotion and disease prevention.

The objectives of this research were: (1) to determine the best combination of roasting process variables for the production of a high antioxidant roasted amaranth flour (RAF) suitable to elaborate a nutraceutical beverage, (2) to produce high antioxidant extruded amaranth flour (EAF) suitable to elaborate a nutraceutical beverage using extrusion process conditions previously optimized, and (3) to produce two nutraceutical beverages from RAF and EAF.

Materials and Methods

Reagents

Dichlorofluorescin diacetate, 2,2′-Azobis (2-amidinopropane), trifluoroacetic acid, were obtained from Sigma Chemical Co. (St Louis, MO). Sodium hydroxide, hexane, methanol, ethanol and ethyl acetate were purchased from DEQ (Mexico). All reagents used were of analytical grade.

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Grains

Amaranth (*Amaranthus hypochondriacus* L) grains var Temoac were purchased in the local market of Temoac, Morelos, México.

Methods

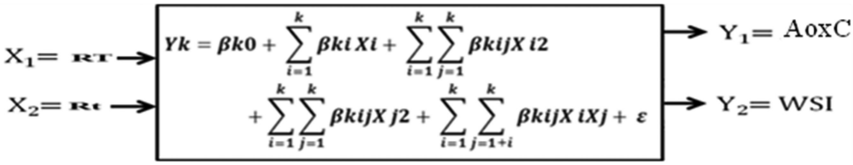
Preparation of Roasted Amaranth Flour (RAF)

Roasting process was carried out in a cylindrical stainless steel roaster with controlled temperature and time and 30 L capacity; the apparatus was built for roasting amaranth grains. Response surface methodology (RSM) was applied to determine the best combination of roasting process variables to produce roasted amaranth flour with high antioxidant capacity and water solubility index. The independent process variables were: Roasting temperature (RT, 110-160°C) and roasting time (Rt, 1-10 min). The dependent response variables chosen were antioxidant capacity (AoxC) and water solubility index (WSI). An experimental central composite experimental design with two factors and five variation levels was chosen; 13 treatments were generated (Table 1). The amaranth grains (1 kg lots) were mixed with lime (0.21 $Ca(OH)_2$/100 g amaranth) and conditioned with purified water to reach a moisture content of 18% w/w. Each lot was packed in a polyethylene bag and stored at 4°C for 8 h. Prior to roasting, the amaranth grains were tempered at 25°C for 4h. After roasting amaranth grains were cooled, equilibrated at environmental conditions (25°C, 65% RH), milled to pass through an 80-US mesh (0.180 mm) screen, and packed in plastic bags. Roasted amaranth flours (RAF) were stored at 4°C until use. The resulting roasted amaranth flours were evaluated for AoxC and WSI.

RSM Experimental Design and Statistical Analysis for Roasting Process

Response surface methodology (RSM) was applied to determine the best combination of roasting process variables to produce roasted amaranth flour with high antioxidant capacity and water solubility index. The independent process variables were: Roasting temperature (RT, 110-160°°C) and roasting time (Rt, 1-10 min). The dependent response variables chosen were antioxidant capacity (AoxC) and water solubility index (WSI). An experimental central composite experimental design with two factors and five variation levels was chosen; 13 treatments were generated (*21*). The following empirical "black box" modeling presents the relationships among the process and response variables.

The expression inside the "black box" represents AoxC, and WSI when the value of k is changed from 1 to 2; β_{ko}, β_{ki}, β_{kii}, and β_{kij} represent the constant and coefficients of linear, quadratic, and interactive effects, respectively; X_i, X_{ii}^2, and X_iX_j represent the linear, quadratic, and interactive effects of the independent variables, respectively, while ε is the random error primarily to account for the inability to determine the true model. Applying the stepwise regression procedure, non-significant terms (p>0.01) were deleted from the second order polynomial and a new polynomial was recalculated to obtain a predictive model for each response variable. The statistical analysis system software Design Expert version 7.0.0 was used for analysis and evaluation (*21*).



$$Yk = \beta k0 + \sum_{i=1}^{k} \beta ki\, Xi + \sum_{i=1}^{k}\sum_{j=1}^{k} \beta kij X\, i2 + \sum_{i=1}^{k}\sum_{j=1}^{k} \beta kij X\, j2 + \sum_{i=1}^{k}\sum_{j=1+i}^{k} \beta kij X\, iXj + \varepsilon$$

Table 1. Experimental design to get different combinations of roasting temperature / roasting time to obtain flours of roasted amaranth. Experimental results to the response variables

STD	*RUN*	*Process variables*		*Response variables*	
		RT	*Rt*	*AoxC*[1]	*WSI*[2]
1	10	117	2.3	5,272	23.2
2	5	117	8.7	5,398	21.8
3	2	153	2.3	6,208	18.6
4	9	153	8.7	6,831	17.6
5	11	135	1	5,765	20.0
6	4	135	10	7,023	19.0
7	12	110	5.5	6,156	22.6
8	13	160	5.5	7,502	16.6
9	7	135	5.5	7,278	19.6
10	8	135	5.5	7,731	21.2
11	6	135	5.5	7,723	19.4
12	1	135	5.5	7,513	20.4
13	3	135	5.5	7,934	19.4

RT= Roasting Temperature (°C); **Rt=** Roasting time (min); **AoxC=** Antioxidant capacity; **WSI=** Water Solubility Index; [1] µmol Trolox equivalent/100 g sample (dw); [2] g / 100 g sample.

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Optimization of Roasting Process

Conventional numeric method was applied as optimization technique to obtain maximum AoxC and WSI values. Predictive models (Table 2) were used to obtain individual desirabilities (Figure 1); which were employed to calculate a global desirability (D) (Figure 2) for observation and selection of superior (optimum) combination of RT and Rt for producing optimized roasted amaranth flour. To perform this operation the Design-Expert program (V 7.0.0) of the STAT-EASE software was utilized (*21*).

Preparation of Extruded Amaranth Flour (EAF)

The procedure recommended by Vargas-Lopez (*22*) and De Queiroz *et. al* (*23*) was optimized by Montoya-Rodríguez (*24*) to obtain extruded amaranth flour with high antioxidant capacity: Amaranth kernels (1 kg lots) were ground in a domestic blender to obtain fine grits that passed through a 40-US mesh (0.074 mm) screen. These fine grits were mixed with lime (0.21 $Ca(OH)_2$/100 g amaranth) and conditioned with purified water to reach a moisture content of 28% w/w. Each lot was packed in a polyethylene bag and stored at 4°C for 12 h. Prior to extrusion, the grits were tempered at 25 °C for 4h. Extrusion cooking was done using single screw laboratory extruder Brabender model 20 DN (CW Brabender Instruments, Inc., South Hackensack, NJ, USA) equipped with a 19 mm screw-diameter; length-to-diameter 20:1; nominal compression ratio 2:1; and die opening of 2.4 mm. The inner barrel was grooved to ensure zero slip at the wall. The barrel was divided into two independent electrically heated feed ends and air-cooled central zones. A third zone, at the die barrel, was also electrically heated but was not air-cooled. Zones 1 were set a at temperatures of 20 and 10°C lower, respectively, compared with the third zone. A screw –operated hopper sent the feedstock into the extruder at 30 rpm. Feed rate was set at 70 g/min. Extrusion temperature, defined as temperature at the die end of the barrel, and screw speed were 125°C and 130 rpm, respectively. Extrudates were cooled, equilibrated at environmental conditions (25°C, 65% RH) for 12 h, milled (UD Cyclone Sample Mill, UD Corp, Boulder, CO, USA) to pass through an 80-US mesh (0.180 mm) screen, packed in plastic bags. The resulting extruded amaranth flour (EAF) was stored at -20°C and 4 °C until use.

Nutritional Content and Physicochemical Properties of the Roasted and Extruded Amaranth Flours

The nutritional content (% of proteins, lipids, ashes and carbohydrates, total dietary fiber (soluble / insoluble) and physicochemical properties (Color, water activity, pH, particle size index, apparent density, water solubility index, oil and absorption index, dispersability) were determined for roasted and extruded amaranth flours. Raw amaranth flour was used as reference. All determinations were made in triplicate.

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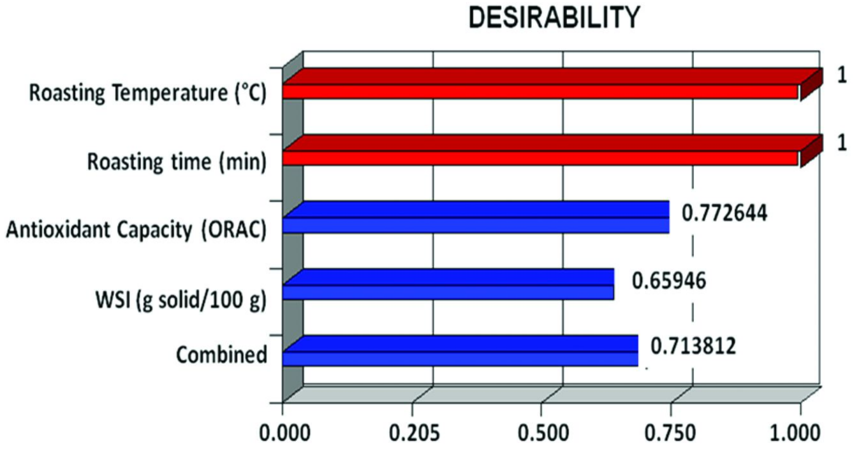

Figure 1. Individual desirabilities obtained with predictive models.

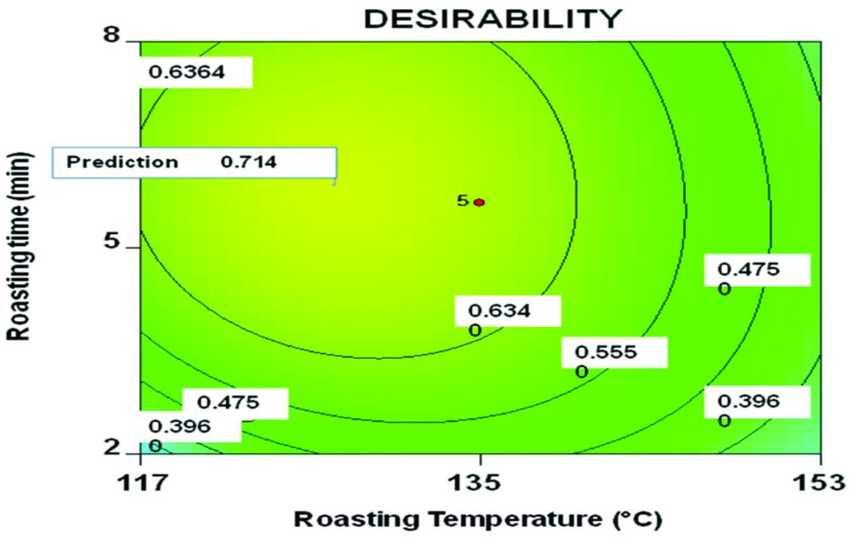

Figure 2. Global desirability obtained with individual desirabilities.

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Table 2. Regression coefficients and analyses of variance of the second order polynomial equations (predictive models) showing the relationships among response (Y_K) and process variables

Coefficients	*Total Antioxidant Capacity (Y_{AoxC})*		*Water Solubility Index (Y_{WSI})*	
	Coded Values	*Uncoded Values*	*Coded Values*	*Uncoded Values*
Intercept				
B	7526.25	-30112.34	20.15	37.18
Linear				
β1	504.93*	493.64*	-2.14*	-0.12*
β2	625.56*	755.72*	-0.38*	-0.14*
Quadratic				
β11	-550*	-1.76*	NS	NS
β22	-624.34*	-84.24*	NS	NS
Interaction				
β12	NS	NS	NS	NS
R^2	0.8841		0.9075	
$P\leq$	0.05		0.05	

NS= Non significant; * Significant ($p\leq0.05$).

Proximate Composition

The following AOAC (*25*) methods were used to determine proximate composition: Drying at 105°C for 24 h, for moisture (method 925.09B); incineration at 550°C, for ashes (method 923.03); defatting in a Soxhlet apparatus with petroleum ether, for lipids (method 920.39C); micro-Kjeldahl for protein (Nx6.25) (method 960.52); and enzymatic-gravimetric methods for total dietary fiber (method 985.29). All determinations were made by triplicate.

Physicochemical Properties

Total Color Difference (ΔE)

The surface color of the samples was measured using a Minolta color-difference meter Model CR-210 (Minolta LTD, Osaka, Japan). The parameters ***L*** (0 = black, 100 = white), ***a*** (+ value = red, - value = green) and ***b*** (+ value = yellow, - value = blue) were recorded. The ***L***, ***a***, and ***b*** values of a white standard (std) tile used as reference were 97.63, 0.78 and - 2.85, respectively. $\boldsymbol{\Delta E}$ was calculated as $\boldsymbol{\Delta E} = ((\boldsymbol{\Delta L})^2 + (\boldsymbol{\Delta a})^2 + (\boldsymbol{\Delta b})^2)^{1/2}$, where $\boldsymbol{\Delta L} = \boldsymbol{L}_{\mathrm{std}} - \boldsymbol{L}_{\mathrm{sample}}$, $\boldsymbol{\Delta a} = \boldsymbol{a}_{\mathrm{std}} - \boldsymbol{a}_{\mathrm{sample}}$, $\boldsymbol{\Delta b} = \boldsymbol{b}_{\mathrm{std}} - \boldsymbol{b}_{\mathrm{sample}}$.

Water Activity (a_w)

This parameter was determined in 5 g flour samples, tempered at 25 °C, using a Hygrometer Aqua Lab Model CX-2 (Decagon Devices Inc, Pullman, WA, USA) which was calibrated with a potassium chloride saturated solution ($a_{\mathrm{w}} = 0.841$ at 25°C). After leaving the samples for 1 hour, the headspace equilibrium was attained and the readings were taken (*26*).

pH

This parameter was measured with a calibrated pH meter Orion Mod 520A (Orion Research Inc, Beverly, MA, USA). Ten grams of each sample were suspended in 100 ml of boiled distilled water (*25*). The slurry was shaken (1,500 rpm, 25°C, 20 min) using an orbital shaker (Cole Parmer Model 21704-10, Cole Parmer International, Vernon Hills, IL, USA).

Particle Size Index (PSI)

Flour samples of 100 g were place in a series of US standard sieves (WS Tyler, Inc, Mentor, OH, USA) with the following sizes: 40=420 µm, 60=318 µm, 80=180 µm, 100=150 µm. Sieves were shaken by a Ro-Tap machine (WS Tyler, Inc, Mentor, OH, USA) for 10 min. The material retained on the sieves was expressed as percent overs. To compute the PSI of flours, the following formula was applied $\mathrm{PSI} = \sum a_i b_i$, where, a_i= percentage on sieve $\boldsymbol{i}$, and b_i=coefficient relative to sieve $\boldsymbol{i}$. The b_i values for sieves numbers 40, 60 and 80 where 0.4, 0.6, and 0.8, respectively. Overs from the sieve number 100 and from the pan were added and an overall b_i=1.0 was assumed (*27*).

Apparent Density (AD)

The ground samples were placed in a known volume stainless cylinder until topped at 25°C. The device was topped five times and the flour density obtained dividing the sample mass by the cylinder volume (*28*).

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Water Solubility Index (WSI)

The WSI was assessed as described by Anderson *et al* (*29*). Each flour sample (2.5 g) was suspended in 30 mL of distilled water in a tared 60 mL centrifuge tube. The slurry was shaken with a glass rod for 1 min at room temperature and centrifuged at 3,000xg and 25°C for 10 min. The supernatant was poured carefully into a tared evaporating dish. The WSI, expressed as g of solids/g of original (2.5 g) solids, was calculated from the weight of dry solids recovered by evaporating the supernatant overnight at 110 °C.

Oil Absorption Index (OAI)

Oil absorption index was determined according to Ordorica-Falomir (*30*). A sample of 0.5 g of flour and 3 mL of vegetable oil were placed into a graduate centrifuge tube. The slurry was shaken in a vortex during 1 min. The sample was incubated at 25°C for 30 min and centrifuged at 1600xg and 25°C for 25 min. Oil volume was measured and the oil absorption index was calculated subtracting this value from the original volume. The result was expressed as mL of absorbed oil / g of flour.

Dispersability

It was determined according to Mora-Escobedo *et al.* (*31*). A flour sample of 1 g was suspended in a graduate conic tube with 10 mL of distilled water and agitated at 1,000 rpm for 5 min, and allowed to settle for 30 min. The volume of settled particles was recorded and subtracted from 10. This value was multiplied by 10 to give the percentage dispersability.

Total Antioxidant Capacity (AoxC)

Extraction of Free Phenolics

Phenolic compounds in amaranth samples were extracted as previously reported by Dewanto *et al.* (*32*) with minor changes. A dry ground of 0.5 g was mixed with 10 mL chilled ethanol-water (80:20, v/v) for 10 min in a shaker at 50 rpm. The blends were centrifuged (3,000xg, 10 min) (Sorvall RC5C, Sorvall Instruments, Dupont, Wilmington, DE, USA) in order to recover the supernatant. The extracts were concentrated to 2 mL at 45°C using a vacuum evaporator (Savant SC250 DDA Speed Vac Plus Centrifugal, Holbrook, NY, USA) and stored at -20°C.

Extraction of Bound Phenolics

Bound phenolics in amaranth samples were extracted according to the method recommended by Adom and Liu (*33*) and Adom *et al.* (*34*). After extraction of free phenolic compounds, the pellet was suspended in 10 mL of 2M NaOH at room temperature and nitrogen was flushed to displace air present in the tube headspace before digestion. The samples were hydrolyzed at 95 and 25°C in a shaking water bath oscillating at 60 rpm for 30 and 60 min, respectively. The hydrolyzed was neutralized with an appropriate amount of HCl before removing lipids with hexane. The final solution was extracted five times with 10 mL ethyl acetate and the pool was evaporated to dryness. Bound phenolic compounds were reconstituted in 2 mL of 50% methanol and stored at -20°C until use.

Antioxidant capacity (AoxC)

Free and bound hydrophilic antioxidant capacities were determined using the oxygen radical absorbance capacity (ORAC) assay; extracts were evaluated against Trolox as standard, with fluorescein as probe as described Ou *et al.* (*35*). Peroxyl radicals were generated by 2,2'-azobis (2-amidinopropane) dihydrochloride, and fluorescent loss was monitored in a Synergy microplate reader (Dynergy TM HT Multidetection, BioTek, Inc, Winooski, VT, USA). The absorbance of excitation and emission was set at 485 and 538 nm, respectively. The antioxidant capacities were expressed as micromoles of Trolox equivalents (TE) per 100 g of dry weight sample.

Beverages Making

Extruded and optimized roasted amaranth flours were used to prepare two beverages: Each flour (110 g) was added to 1 L purified water with fructose (13 g), powdered cinnamon (3 g), and powdered vanillin (7 g); the suspension was stirred in a domestic shaker (medium velocity), refrigerated (8-10°C) and sensory evaluated for acceptability (*A*). All determinations were made by triplicate.

Sensory Evaluation

The beverages (RAF Beverage, prepared with roasted amaranth flour; and EAF Beverage, prepared with extruded amaranth flour) were made and evaluated every day. The beverages were evaluated after 30 min of preparation, at room temperature. Sensory evaluation of each beverage was done using a panel of 80 judges (semi-trained panellists). The judges were seated in individual booths in a laboratory with controlled temperature (25°C) and humidity (50-60%), and daylight fluorescent lights. Samples were evaluated for acceptability using a hedonic scale of 9 points, where 9 means like extremely, and 1means dislike extemely (*36*).

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Results and Discussion

Predictive Model for Antioxidant Capacity (AoxC) of Roasted Amaranth Flour

The AoxC of the roasted amaranth flours varied from 5,272 to 7,934 µmol TE / 100 g dry flour (Table 1). Analysis of variance showed that AoxC was significantly dependent on linear terms of roasting temperature (RT ($p \leq 0.05$)), roasting time (Rt ($p \leq 0.05$)), quadratic terms of RT, and Rt (RT ($p<0.05$); Rt ($p<0.05$)):

Using coded values:

$$Y_{AoxC} = 7526.25 + 504.93X_1 + 625.56X_2 - 550X_1^2 - 624.34X_2^2$$

Using original values:

$$Y_{AoxC} = -30112 + 493.64RT + 755.72Rt - 1.76RT^2 - 84.24Rt^2$$

The predictive model explained 88.41% of the total variation ($p \leq 0.05$) in AoxC values (Table 2).

Predictive Model for Water Solubility Index (WSI) of Roasted Amaranth Flour

The WSI of the roasted amaranth flours varied from 16.6 to 23.2 g solids/ 100 g original solids (Table 1). Analysis of variance showed that WSI was significantly dependent on linear terms of roasting temperature (RT ($p \leq 0.05$)), and roasting time (Rt ($p \leq 0.05$)):

Using coded variables:

$$Y_{WSI} = 20.15 - 2.14X_1 - 0.38X_2$$

Using Original values:

$$Y_{WSI} = 37.18 - 0.12RT - 0.14Rt$$

The predictive model explained 90.75% of the total variability ($p \leq 0.01$) in WSI values (Table 2).

Optimization of Roasting Process for Amaranth Grain

Predictive models of each one of the response variables allowed to obtain individual desirabilities (Figure 1) which were utilized for calculating a global desirability (D) (Figure 2). The common maximum values for the two response variables were obtained at a D=0.714, as a result of the best combination of roasting process variables for the production of roasted amaranth flour: RT=127°C / Rt= 5.72 min. The D value obtained was between the values that considered to be acceptable (0.6<D<0.8) according to De la Vara and Domínguez (*37*).

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Table 3. Chemical composition, physicochemical, nutritional properties and antioxidant capacity of raw, extruded and roasted amaranth flours

Properties[1]	*Raw Amaranth flour*[3]	*Roasted Amaranth Flour*[3]	*Extruded Amaranth flour*[3]
Chemical composition (%, DW)			
Protein	15.63^{b}	14.59^{a}	15.77^{b}
Lipids	7.86^{a}	6.80^{b}	6.15^{c}
Ashes	3.15^{b}	3.60^{b}	3.50^{a}
Carbohydrates	73.34^{b}	75.01^{c}	74.58^{a}
Total Dietary Fiber	14.62^{a}	13.91^{b}	12.60^{c}
Soluble	4.72^{a}	4.62^{a}	3.20^{b}
Insoluble	9.90^{a}	9.29^{b}	9.40^{b}
Physicochemical			
Color			
Hunter "L"	79.70^{a}	82.12^{a}	75.29^{b}
ΔE	20.90^{b}	26.06^{c}	25.55^{a}
a_w	0.43^{a}	0.41^{c}	0.40^{b}
pH	6.90^{b}	7.30^{b}	7.40^{a}
PSI (%)	42.05^{a}	37.59^{b}	21.40^{c}
AD (g/cm^3)	0.52^{b}	0.58^{c}	0.57^{a}
WSI	13.42^{b}	21.20^{c}	60.80^{a}
Oil Absoprtion[2]	1.45^{a}	1.32b	1.35^{b}
Dispersability (%)	34.00^{a}	36.00a	28.8^{b}
Nutraceutic			
Total Antioxidant Capacity	$4{,}403^{c}$	$7{,}053^{a}$	$5{,}046^{b}$
Free Phenolics	$1{,}947^{b}$	3,113a	$1{,}467^{c}$
Bound Phenolics	$2{,}456^{c}$	$3{,}940^{a}$	$3{,}579^{b}$

[1] **ΔE** = Total Color Difference; **a_w** = Water Activity; **PSI=** Particle Size Index; **AD=** Apparent density; **WSI=** Water Solubility Index (g solid /100 g); [2] mL oil/g flour; [3] The comparison was made by lines using Duncan's multiple range test. Values with different letters are significantly different ($p \leq 0.05$).

Nutrimental Content and Physicochemical Properties of Roasted and Extruded Amaranth Flours

Raw and extruded amaranth flours had similar ($p \leq 0.05$) protein content (15.63-15.77%, dw) while optimized roasted amaranth flour showed the lowest

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($p \leq 0.05$) protein content (14.59%, dw) (Table 3). Other researchers (*38*, *39*) have reported similar protein content for extruded amaranth flour. The lowest protein content of roasted amaranth flour might be associated to formation of Maillard reaction products (*40*). The raw amaranth flour showed the highest lipid content (Table 3); these results are in agreement with those reported by other authors (*8*, *38–40*). The amaranth grains contain high starch content (60-70%) which is able to create complexes with lipids during roasting and extrusion processes. The total dietary fiber (TDF) contents of raw, roasted and extruded amaranth flours were 14.62, 12.60, and 13.91 %, dw, respectively (Table 3). Reynoso-Camacho *et al* (*41*) observed that the pattern of reduced colon cancer in Sprague-Dawley rats was influenced by the presence of dietary fiber; roasted and extruded amaranth flour by the TDF content could be considered as functional foods.

The roasted and extruded amaranth flours had higher ($p<0.05$) ashes content than raw amaranth flour (3.50-3.60 vs. 3.17%, dw) (Table 3); this behavior is related with the addition of lime during roasting and extrusion processes. The raw amaranth flour showed lower ($p<0.05$) ΔE than roasted and extruded amaranth flours (20.90 vs 25.55-26.06) (Table 3); higher ΔE indicate darkerflours.

The water activity of raw and processed amaranth flours varied from 0.40 to 0.43 (Table 3); in this range the development of enzymatic activity, growth of microorganisms and chemical reactions are restricted, meaning a long shelf-life. The apparent density (AD) of the amaranth flours varied from 0.52 to 0.58 g/cm^3; the lowest and highest AD corresponded to raw and roasted/extruded amaranth flours, respectively (Table 3). The WSI of raw, roasted, and extruded amaranth flour were 13.42, 21.2, and 60.80 g solids/100 g original solids, respectively (Table 3); both process roasting and extrusion increased ($p<0.05$) the WSI of amaranth flour. Vargas-López *et al* (*42*) evaluated the effect of extrusion on WSI of amaranth flour, they reported WSI values of 1.27-15.87 g solids/100 g original solids for extruded amaranth flour; these values are lower than those found in this research. González *et al.* (*43*) suggest that maximum WSI values are reached when the amaranth is processed by extrusion because of its endosperm structure is softer than the other cereals.

Antioxidant Capacity (AoxC) of Roasted and Extruded Amaranth Flours

Table 3 shows the total hydrophilic antioxidant activity (sum of antioxidant capacities of free and bound phenolic compounds) or ORAC values of amaranth, roasted, and extruded amaranth flours. Processing of the whole raw grains increased ($p<0.05$) the total ORAC value of the roasted and extruded amaranth flours when compared with the unprocessed mixture (5,046-7,053 vs. 4,403 µmol Trolox equivalent (TE)/100 g sample (dw)) (Table 3). The antioxidant capacity of roasted amaranth flour was higher than raw and extruded amaranth flour (7,053 *vs.* 4403-5046 µmol TE/100 g, dw). Assunta *et al.* (*44*) reported that the increase on the *AoxC* after roasting process is associated to the formation of melanoidins of low molecular weight.

It was also observed that the ORAC value of free phenolic compounds significantly increased ($p<0.05$) and ORAC value of bound phenolic decreased ($p<0.05$) in extruded amaranth flour (Table 3). This behavior could be attributed

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to (i) breaking of conjugated phytochemicals and release free phytochemicals (*32*), (ii) prevention of enzymatic oxidation and, (iii) darker colors of the roasted and extruded flours indicating formation of Maillard reaction products having antioxidant properties (*45*).

Our results show that the bound phenolics were the primary contributors (56-71%) to ORAC value in raw, roasted and extruded amaranth flours (Table 3). Bioactive phytochemicals exist in free, soluble-conjugated, and bound forms; bound phytochemicals, mostly in cell wall materials, are difficult to digest in the upper intestine and may be digested by bacteria in the colon to provide health benefits and reduce the risk of colon cancer (*33*, *46*).

Nutritional Content, Antioxidant Capacity, and Acceptability of the Beverages

The formulation of a 200 mL portion of the two beverages prepared from roasted and extruded amaranth flours (Table 3) was based on those of traditional beverages widely consumed in Mexico, which are produced from different grain flours (for example, rice, barley), as well as sensorial tests to define the proper amounts for each ingredient (data not shown). The Mexican norm NMX-F-439-1983 for foods and non-alcoholic beverages was also considered. This norm defines a nutritious beverage when it contains at least 1.5% protein or protein hydrolyzates with a quality equivalent to that of casein; it also establishes that the beverage must contain 10 to 25% of the main ingredient used to prepare it; these beverages can also contain up to 2% ethanol, sweeteners, flavoring agents, carbon dioxide, juices, fruit pulp, vegetables or legumes and other additives authorized by the Health and Assistance Secretary of Mexico. The formulations used in this research contained 11% of the amaranth flours and 1.50-1.57% proteins of good quality. Besides, these beverages contained fructose for two reasons: 1) to satisfy the recommendations of the Health and Assistance Secretary of Mexico, regarding the fact that a 200 mL portion of a beverage (food) must contain no more than 100 Cal, and 2) to maintain a high sensorial acceptability.

The 200 mL portion of the beverages prepared with 22 g of the roasted or extruded amaranth flours contained 3.01-3.13 g proteins, 1.22-1.35 g lipids, 14.1-14.3 g carbohydrates and 80 Cal (Table 3). This portion covers 23.1-24.9 % and 15.5-16.7% of the daily protein requirements for children 1-3 and 4-8 years old, respectively. The nutraceutical beverages (200 mL) from roasted and extruded amarnanth flours showed a total antioxidant activity of 1,400 and 1,000 µmol TE, respectively (Table 3); which contributes with 28-47% and 20-33% of the recommended (3,000 to 5,000 µmol TE) daily intake for antioxidants (*47*), respectively. The semi-trained panelists assigned an average value of 8.1 and 8.4 in acceptability to the beverages from roasted and amaranth flours, respectively (level of satisfaction between “I like it” and “I like it extremely”) (Table 3). It is expected that this acceptability allows an adequate consumption to provide health benefits.

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Conclusions

The best combination of roasting and extrusion process variables for the production of roasted and extruded amaranth flours to elaborate a beverage with high antioxidant capacity and acceptability were: (1) Roasting temperature = 127°C / Roasting time = 5.5 min, and Extrusion temperature= 125°C / Screw velocity = 130 rpm, respectively. The roasted and extruded amaranth flours had an antioxidant activity of 7,053 and 5,046 µmol Trolox equivalents/ 100 g sample (dw), respectively. A 200 mL portion of the beverages from roasted and amaranth flours contributes with 28-47% and 20-33% of the recommended daily intake for antioxidants, respectively. Both beverages were evaluated with an average acceptability of 8.1-8.4 (level of satisfaction between "I like it" and "I like it extremely"). The high nutritional, antioxidant and sensory value of the beverages can be attributed at least partially to the application of the optimum roasting and extrusion processing conditions. The nutraceutical beverages could be used for health promotion and disease prevention as an alternative to beverages with low nutritional / nutraceutical value.

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Chapter 14

Bean Concentrates and Inflammation Reduction

M. Oseguera-Toledo,[1] V. P. Dia,[2] E. Gonzalez de Mejia,[2] and S. L. Amaya Llano*,[1]

[1]Programa de Posgrado en Alimentos del Centro de la República (PROPAC), Universidad Autónoma de Querétaro
Apdo. 184 Querétaro Qro. 76010, México
[2]Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801
***E-mail: samayal@uaq.mx**

The objectives of this study were to evaluate the effect on markers of inflammation in lipopolysaccharide (LPS)-induced RAW 264.7 macrophages of protein hydrolysates of the common bean (*Phaseolus vulgaris* L.) varieties Negro 8025 and Pinto Durango. Cell viability was determined and the percentage of viable cells was calculated and concentrations that allowed >80% cell viability were used to determine the effect on markers of inflammation. Alcalase hydrolysates of common beans inhibited cyclooxygenase-2 expression, prostaglandin E_2 production, inducible nitric oxide synthase expression and nitric oxide production, for Pinto Durango, with IC_{50} values of 34.9 ± 0.3, 13.9 ± 0.3, 5.0 ± 0.1 and 3.7 ± 0.2 µM, respectively while var. Negro needed 43.6 ± 0.2, 61.3 ± 0.3, 14.2 ± 0.3 and 48.2 ± 0.1 µM. In conclusion, hydrolysates from common beans can be used to combat inflammatory associated diseases.

Introduction

Common Bean General Aspects

Plant proteins provide nearly 65% of the world protein supply for humans (*1*) were legumes are very important in the traditional diet of many places throughout the world (*2*). Legumes contain high levels of proteins ranging from 20 to as much

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ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

as 40 g/100 g dry matter. Legume include peas, beans, lentils, peanuts, and other podded plants that are used as food. The nutritional profile of beans shows that they are high in protein, low in saturated fat and high in complex carbohydrates and fiber (*2*). The dry bean consumption from an average U.S diet is about 3 kilograms per capita per year, while in Mexico is about 11 kilograms, however in some rural communities with less than 2500 inhabitants, they are consuming about 21 kilograms per capita per year.

In the common bean origin, two major ecogeographical gene pools are recognized: Mesoamerica (from Central America, Mexico and Colombia) and the Andes (from southern Peru, Bolivia and northern Argentina (*3*, *4*).

The wild common bean geographical distribution comprises the north of Mexico to the north of Argentina. Domesticated common beans show differences in morphology, isozymes, phaseolins, and nuclear, chloroplast and mitochondrial DNA markers, which parallel those found in wild Mesoamerican and Andean beans (*3*).

Mexico produces about 50 different varieties of dry beans and they are divided into four principal groups which are: black bean, pinto bean, pink bean and yellow bean (*5*). Figure 1 shows the four principal varieties of beans found in Mexico.

Common bean (*Phaseolus vulgaris* L) is a good source of protein (16–33%), some vitamins, minerals, carbohydrates and fiber. Diverse legume varieties contains different protein profiles and properties, which could impact bioactivity, functionality and further affect applications in the food industry.The main proteins in beans are classfied as salt-soluble globulins, including a prevalent fraction of vicilin sometimes also named as phaseolin or G1 globulin and a small fraction of legumin (*6*). Phaseolin, represents 40-50% of the total seed protein. Its low sulphur- amino acid content (notably methionine) and tryptophan means low nutritional quality for man and other animals. Furthermore, raw phaseolin is highly resistant to proteolytic hydrolysis (*7*). Utilization of raw legume protein has however a lower potential, partly due to deficiency of some of the essential amino acids in these proteins, and also due to the presence of antinutritional factors associated with these proteins. In order to improve the nutritive value a number of methods can be applied. Enzymatic hydrolysis of proteins has been applied to improve functional properties because of great potential (*8*).

Bean Hydrolysates Obtention

Protein hydrolysates are derived from several sources and can be classified according to their degree of hydrolysis (DH). The DH is defined as the percentage of hydrolyzed peptide bonds. Protein hydrolysates with low degree of hydrolysis (<10%) often have superior functional properties than the original proteins and are used as food ingredients. Protein hydrolysates with different degrees of hydrolysis are used as flavorings agents. Extensive protein hydrolysates (DH>10%) are used as protein supplements or in special medical diets, such as hypoallergenic foods. Protein hydrolysates for food applications are usually produced by limited enzymatic hydrolysis of protein molecules in foodstuff, yielding polypeptides that are smaller in molecular mass.

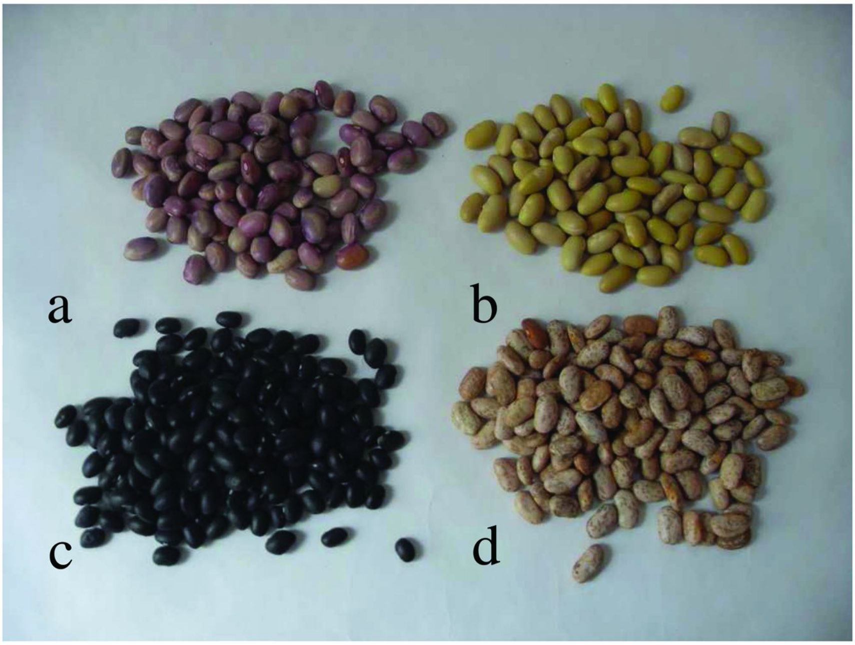

Figure 1. Principal varieties of beans found in Mexico: (a) pink bean, (b) yellow bean, (c) black bean, (d) pinto bean.

Enzymatic hydrolysis can be controlled by varying the ratio of enzyme to substrate, hydrolysis time and temperature. One of the important factors is the choice of enzymes since different enzymes have different specific activities and optimal working parameters. Different proteases, such as pepsin, pancreatin, trypsin, or chymotrypsin, have been used to hydrolyse protein to produce peptides possessing special bioactivities. Alcalase is an alkaline enzyme produced from *Bacillus licheniformis* with rising use for industrial hydrolysate production because of its broad specificity endoprotease activity, low cost and high tolerance for alkaline pHs (*8*). It has optimal pH range at the value of 8 to 10 that could reduce the risk of microbial contaminations, with an optimum temperature that ranged from 50 to 70°C.

Bean Hydrolysates Properties

Numerous studies have demonstrated that protein hydrolysates from enzymatic hydrolysis of animal and proteins can act as direct scavengers of diverse free radicals or behave as antioxidants in model systems (*9*).

Demand of Functional and Nutraceutical Foods

The functional food and nutraceutical industry represents over $75.5 billion in US industry with predictions of growing to $167 billion in 2010 (*10*). Nutraceuticals can play an important role in controlling a number of related diseases associated with unhealthy lifestyle hence an increasing percentage of . people are turning to nutraceuticals (*11*). Natural products found in plants have been recognized as prophylactic agents for the health maintenance and diseases prevention. Functional foods provide an opportunity to improve the health beyond their nutritional properties. The frontier between nutraceuticals and functional foods is not completely clear being the focal distinction the format in which they are consumed (*12*). Nutraceuticals are sold in the form of prepared products like pills, capsules, powder, etc. They are medicinal formula, which are not associated generally with the foods and can exert a beneficial physiological effect, or assure protection against chronic diseases, while functional food are always consumed as ordinary foods. Conventional dietary recommendations have focused on the consumption of fruits, vegetables, legumes and whole grains and to eat less added sugar and solid fat as a way to prevent chronic diseases (*13*). The potential health benefits of functional foods and nutraceuticals are attributable to the presence of specific functional compounds and their molecular derivatives released during digestion. Some legume plants are a source of various bioactive peptides which are produced by microbial fermentation and/or enzymatic digestion. They have shown functionalities as antioxidative, antimicrobial, antihypertensive, cytomodulatory and immunomodulatory effects (*14*).

Inflammation

Inflammation Definition and Importance

Inflammation is the first response to combat infection and tissue injury that serves to activate tissue repair mechanisms. The pathogen invasion or cell damage are often detected by pattern recognition receptors, mainly expressed by cells that participate in the innate immune response such as macrophages, fibroblasts, leukocytes and neutrophils. These receptors detect patterns such as microbial nucleic acids, lipoproteins, and carbohydrates. Once such receptors are triggered, signaling cascades are transmited that activate transcripction factors that regulate genes that amplify the initial inflammatory response (*15*, *16*).

The control of the innate and adaptative immune systems is required for normal tissue homeostasis and effective responses to infection and injury, otherwise these processes can lead to chronic inflammatory diseases that can be developed in many organ systems. During inflammatory response vascular alteration plays an important role, blood flow is increased and brings in circulating leukocytes. Factors such as histamine, prostaglandin and nitric oxide promote vasodilation. Activated innate immune cells remove foreign particles and host debris by phagocytosis, and they also secrete cytokines that activate the adaptive immune response (*15*).

If successful, acute inflammation is resolved, restoring normal tissue or forming tissue scars. If the stimulus is not eliminated then the inflammatory process will continue and evolve (*17*). Inflammation also participates in several chronic diseases as shown in Figure 2 (*18*, *19*).

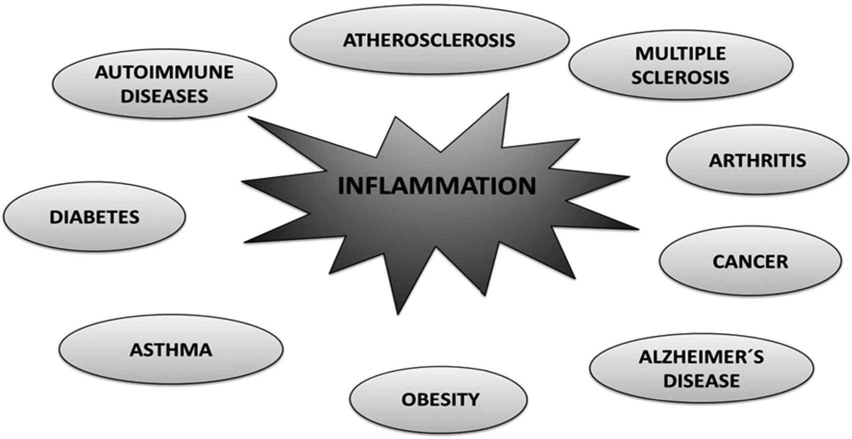

Figure 2. Participation of inflammation in chronic diseases.

Inflammation can be measured by the use of different markers such as interleukins (IL), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-KB), tumor necrosis factor-α (TNF-α), cyclooxygenase (COX) and nitric oxide (NO) among others (*20*, *21*).

As it was mentioned before chronic inflammation has been linked with cancer, particularly adult carcinomas. In addition, tumor associated inflammation drives tumor development through the recruitment of leukocytes, and the production of cytokines and chemokines (*22*, *23*).

Obesity is also associated with an inflammation process in adipose tissue, characterized by abnormal adipokine production, and the activation of some pro-inflammatory signaling pathways, resulting in the induction of several biological markers of inflammation (*24*).

It is also known that nuclear transcription factors such as nuclear factor kappa B are involved in atherosclerosis. The transcription factors promote gene expression of pro-inflammatory proteins vital for the development of the atheromatous plaque (*25*).

In Hispanic Foods: Chemistry and Bioactive Compounds; Tunick, M., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

Inflammation Markers

To measure inflammation, several indicators or markers have been used such as interleukins, tumor necrosis factor, cyclooxygenase, nitrites, etc.

Interleukins (IL)

Interleukins are cytokines, secreted proteins or signaling molecules, that affect among other processes of inflammation. During inflammation interleukins are released by activated macrophages and activates other inflammatory cells or inhibit some of their functions. If there is a failure in the coordination of cytokines it leads to inflammatory disorders (*26*).

It has been found that interleukins are involved in activation of T lymphocytes (IL-1, 2, 4, 6 and 7), B lymphocytes (IL-1, 2, 4, 5, 6 and 7), and macrophages (IL-1 and IL-4). IL-1 can be promoted by different signals such as microbial products and it plays an important role during inflammation. IL-1 as well as microbial products induces the production of interleukin-6 and interleukin-8. IL-6 also plays a role in inflammation. IL-1 often acts with Tumor Necrosis Factor (TNF) during the pro-inflammatory process. IL-3 increases the inflammatory response by promoting the production of IL-1 and TNF (*27*, *28*).

In inflammatory diseases IL-12 and IL-18 are also produced and affect the production and cellular response to IL-1 and TNF. Because of their multiple pro-inflammatory properties, these cytokines contribute to disease. However in models of inflammation where several cytokines are produced, specific blockade of either IL-1 or TNF or both results in a reduction in inflammation (*27*).

TNF-α

TNF-α (tumor necrosis factor alpha) is a cytokine associated with a variety of inflammatory diseases. It causes cellular responses that are related with inflammation, immune response, cell survival, proliferation and apoptosis (*29*).

TNF-α is mainly produced by macrophages, neutrophils and monocytes and its function is to regulate the immune response to inflammation. TNF-α stimulates the production of inflammatory cytokines. In a study using genetically modified animals and poor of this factor, showed that TNF-α is linked to inflammation, actively participates in the events of early inflammation of tumors (*30*).

Once TNF binds to TNF receptors (TNFR), it triggers a transduction cascade leading to a series of protein-protein interactions and kinase activation, and leads to the translocation of transcription factors other than within the nucleus and induce transcription of inflammatory genes.The activation of TNFR triggers two main kinase pathways: MAPK activation and IKK activation (*31*).

Cyclooxygenase

In the 1990's the discovery of cycloxygenases (COX) was made, also known as prostaglandin H synthase or prostaglandin endoperoxide synthase, is a bifunctional enzyme that attached to the membrane, catalyzes the limiting step in the production of prostaglandins (PGs) and thromboxane (TX) (*32*, *33*).

There are 2 major isoforms known. The isoform COX-1 is considered constitutive and regulates gastrointestinal, renal, vascular and other physiological functions, and the isoform COX-2 an inducible form regulates production of PGs involved in inflammation, pain and fever. The discovery of the COX isoforms led to establishing their importance in inflammatory pathogenesis, including cancer, Alzheimer's and other neurodegenerative diseases.

The first step in the formation of PGs is the liberation of arachidonic acid (AA) coupled with membrane phospholipids. Once released, both COX-1 and COX-2 make it the PGG_2 and PGH_2 by identical reactions: COX is an enzyme that adds two molecules of oxygen to AA to form PGG2 and then reduced to this hydroperoxide highly cyclical in endoperoxide PGH2 reagent (*32*, *33*), which acts as an intermediary substrate for the synthesis of PGs.

The most notable differences between COX-1 and COX-2 can be seen at the level of expression and tissue distribution, as well as cellular and physiological function. Thus, COX-1 is expressed constitutively in most tissues of different animal species, including the gastrointestinal system, liver, kidney, vascular smooth muscle and platelets (*34*, *35*). COX-2, in contrast, in normal situations is undetectable in most tissues and cells (except in the kidney, placenta, brain and platelet, in which there is a basal constitutive expression), but expressed in response to inflammatory process. In particular, overexpression of COX-2 has been observed in the presence of mitogenic and inflammatory stimuli such as lipopolysaccharide (LPS), IL-1, TNF, serum, retinoic acid, etc. (*36*).

Nitric Oxide

Nitric oxide (NO) is a biological molecule involved in a multitude of physiological and pathological processes, such as the regulation of blood pressure, neurotransmission, antimicrobial activity, redox regulation and apoptosis (*37*).

Nitric oxide synthase is an enzyme that produces nitric oxide. In mammals, there are three different isoforms of nitric oxide synthase (NOS), known as the inducible (iNOS), the neuronal (nNOS) and endothelial (eNOS). Only the iNOS is capable to produce high concentrations of NO. This isoform is inducible, in a large number of cells involved in inflammatory processes by cytokines and other pro-inflammatory agents. In contrast nNOS and eNOS are constituents and produce small amounts of NO (*38–41*).

Studies have shown that inflammation in various tissues is accompanied by a high expression of inducible nitric oxide synthase (iNOS) which is capable of producing an excess of NO for a long period of time. Therefore, chronic inflammation has been linked to cancer processes. An increase in the expression of iNOS has been found in human cancer cells in various tissues such as prostate, mouth and esophagus and less in the stomach, colon and breast (*42*).

Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells (NF-κB)

The nuclear factor that binds to the kappa-light chain enhancer of B cells (NF-κB) is a transcription factor that regulates the expression of a variety of cellular genes, involved in many processes such as immune and inflammatory responses, cell adhesion, differentiation, oxidative stress responses, and apoptosis. Which are active in a number of diseases, including cancer, arthritis, chronic inflammation, asthma, neurodegenerative diseases, and heart disease (*43*, *44*).

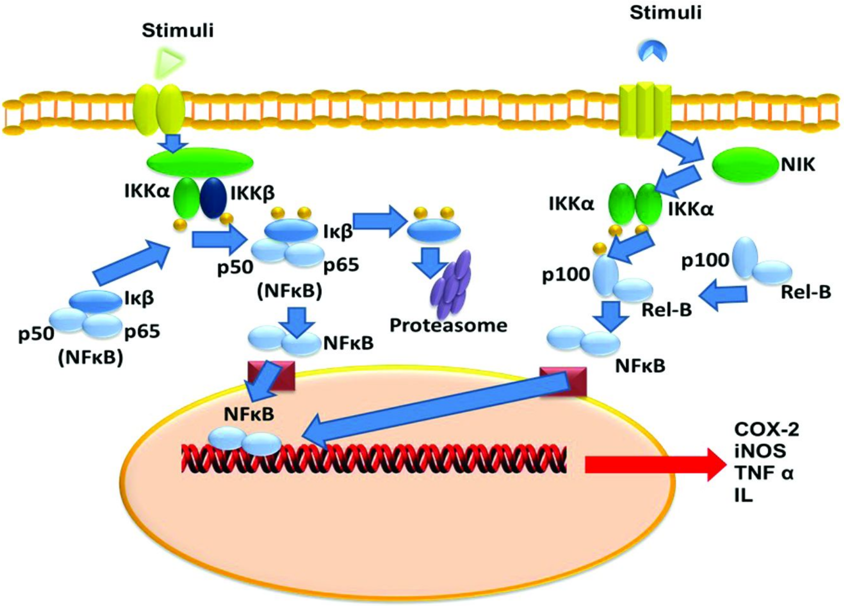

Figure 3. NF-κB Signaling Pathways. IKK activation results in IκB phosphorylation and degradation in the canonical pathway or p100 processing to p52 in the noncanonical pathway. Phosphorylated NF- κB dimers bind to κB DNA elements and induce transcription of target genes.

NF-κB can be activated by a great variety of substance including viruses and their transactivator proteins, T cell mitogens, the cytokines TNF and IL-1, lipopolysaccharide, activators of protein kinase C, etc. The inducible cytoplasmic form of NF-κB contains three protein subunits: a 50 kd (p50) and 65 kd protein subunit (p65), both of which are also found in the active nuclear form and a third subunit inhibitor of kappa B(IκB), which is confined to the cytoplasmic form. IκB inhibits translocation of p50 and p65 subunits in the nucleus thereby preventing DNA binding of NF-κB (*44*).

NF-κB signaling consists of a series of positive and negative regulatory elements. Inducing stimuli trigger IKK activation leading to phosphorylation, ubiquitination, and degradation of IκB proteins as shown in Figure 3. Then NF-κB dimers are released and activated through modifications and translocated to the nucleus where they bind to specific DNA sequences and promote transcription genes. The NF-κB family of transcription factors consists of five members, p50, p52, p65 (RelA), c-Rel, and RelB, encoded by NFKB1, NFKB2, RELA, REL, and RELB, respectively (*45*).

Immunomodulatory Peptides as Therapeutics

Nowadays there is an increase in interest of diet and health, because of that functional foods and nutraceuticals has increased in response of that demand. The therapeutic potentials of functional foods and nutraceuticals are related to specific functional groups and molecular derivatives released during food metabolism (*14*).

Food proteins not only supply calories and amino acids, but also offer health benefits *in vivo* and *in vitro* either in the intact form or as hydrolysates because of their bioactive properties. Peptides with biological functionalities produced *in vitro* or *in vivo* after hydrolysis are called bioactive peptides (*46*, *47*). Biological functionalities include antioxidative, antimicrobial, antihypertensive, cytomodulatory and immunomodulatory effects (*48*, *49*).

Usually bioactive peptides are formed by 3–20 amino acid residues. Although the roles of immunology cells in the immunostimulatory activities of peptides have been studied for several years, the mechanisms by which the immune system is affected is not clear and need further studies (*50*).

Nisin is a peptide bacteriocin and it is produced by *Lactococcus lactis* in order to eliminate other competing gram-positive bacteria. Because of that it has been widely used as a safe food preservative. Nisin also influences some human immune system cells (*51*).

Also it has been suggested that intake of wheat gluten hydrolysate might augment natural killer cell activity although the mechanism is not clear yet (*52*).

Casein hidrolysates (CHs) have shown potential in immunomodulatory. It has been demonstrated the capability of hydrolysates to modulate the activation of transcription factor NF-κB in Caco-2 cells. As it was mentioned before activated NF-κB is related with inflammatory cascade. CHs, produced after digestion with lactic acid bacteria (LAB) proteinases, were tested for their ability Casein hydrolysates that decrease basal NF-kB were produced by digestion with *Lactobacillus helveticus* MIMLh5 and *Lactobacillus acidophilus* ATCC 4356 LAB proteinases (*53*).

The hydrolysis of milk have shown to have intestinal anti-inflamatory activities, two of them are transforming growth factor-β (TGF-β) and casein macropeptide, also known as glycomacropeptide. Furthermore, TGF-β is added to formulas intended for patients with inflammatory bowel disease (*54*). Also, peptides released from enzymatic hydrolysis of certain egg proteins could produce modulatory effects on immune functions. These suggest their potential use in the management of immune disorders (*49*). Delivery of functionally relevant concentrations of biologically active peptides into cells is a challenge because they must locate a cell, traverse the cell membrane, gain access to the cell cytoplasm, and then find its target before the peptide is modified or degraded by extracellular or intracellular enzymes like proteases or lysosomes. One therapeutic method for enhancing cellular uptake is through the use of cell-penetrating peptides, which can be used to deliver a variety of cargo intracellularly. Cell-penetrating peptides are taken up by a variety of cell types rapidly, an essential characteristic when delivering a therapeutic to the multiple cell types involved in chronic inflammation (*55*).

Synthetic peptides corresponding to linear human leukocyte antgen sequences are effective immunoregulators. These peptides offer the opportunity to develop a new class of immunotherapeutics. Some inducing and maintaining immunologic tolerance. Important issues regarding exact mechanisms of action, routes of administration, bioavailability, and the potential for combination therapies require further evaluation (*56*).

Natural thymic peptides have been isolated from calf thymus, Thymalin, was put is recognized as immunocorrector. One of the immunomodulatory molecules (Glu-Trp) has been isolated from Thymalin by reversed-phase high performance liquid chromatography. Thymic like peptides that contain the sequence Glu-Trp indicates the participation in regulating mechanisms of inflammatory processes as cytokine antagonists (*57*).

Effect of Common Bean Hydrolysates on Inflammation Reduction

It has been demonstrated that proteins and peptides from egg, milk, soy, and plant sources have been shown to have anti-inflamatory properties. Oseguera-Toledo et al. (*58*) observed that protein profiles of common bean concentrates of two bean varieties showed strong signals between 43 and 50 kDa and between 20 and 25 kDa. The signals observed between 43 and 50 kDa belong to phaseolin, the main storage protein of common bean (*59*, *60*). During hydrolysis of common bean concentrates using alcalase, it was observed that phaseolin was hydrolysed as time progresses until it almost disappeared at 180 min of hydrolysis. Due to the importance of common bean as a good source of protein in the Mexican diet, attention is being given to its composition and bioactive properties. There are some studies relating the beneficial effect of whole bean and reduction of chronic diseases related to inflammation such as colon cancer (*61–63*) and diabetes mellitus (*64*).

Oseguera-Toledo et al. (*58*) studied the effect of common bean alcalase hydrolysates derived from two cultivars, Negro 8025 (N) and Pinto Durango (P), on NO and PGE_2 production as well as iNOS and COX-2 expression in

LPS-induced RAW 264.7 macrophages. Also, the antioxidant capacity and the possible mechanism of action involved on the antiinflammatory property of the protein hydrolysates were studied. The bean hull was manually removed and the whole beans were ground to produce flour with a particle size equivalent to 20 mesh. Common bean protein concentrate was produced according to Valentas, Levine, and Clark (*65*) with minor modifications. Common bean protein concentrate was hydrolysed by treatment with alcalase at 50 °C and pH 8.0. Hydrolysis was allowed to run for 10, 20, 80, 120 and 180 min with temperature and pH kept constant. The hydrolysates were centrifuged (14000g for 30 min at 4 °C) to remove any precipitates; the liquid hydrolysates were filtered using stirred ultrafiltration cell (Millipore #5123 with 3 kDa membrane) to remove salts from the mixture.

The antioxidant capacity of common bean hydrolysates was evaluated with the method of the oxygen radical absorbance capacity (ORAC) assay (*66*). ORAC assay was determined by measuring the protection of the common bean hydrolysates on fluorescein in the presence of free radicals generated by 2,20-azobis(2-amidinopropane) dihydrochloride (AAPH). The antioxidant capacity of the acid soluble hydrolysates of both varieties showed a maximum antioxidant capacity at 80 min of hydrolysis; the antioxidant capacity increased during the hydrolysis until 80 min and then it started to decrease. Reactive oxygen species and reactive nitrogen species are the most common products of oxidative stress and pro-inflammatory molecules such as IL-1β, TNF-α and lipopolysaccharide can produce oxygen free radicals which can promote the process of oxidation.

Oseguera-Toledo et al. (*58*) also showed for the first time the anti-inflammatory properties of common bean hydrolysates through inhibition of prostaglandin E_2/cyclooxygenase-2 and nitric oxide/inducible nitric oxide synthase pathways in lipopolysaccharide-induced RAW 264.7 macrophages through inhibition of NF-κB pathway.

RAW 264.7 macrophage cell line was cultured and treated with common bean hydrolysates (0.5–200 μM) and LPS. After treatments, nitrite was measured as an indicator of NO production in culture supernatant. The amount of NO was calculated using sodium nitrite standard curve. The common bean alcalase hydrolysates showed inhibition in the production of NO, treatment with LPS increased the expression of iNOS and increased production of NO, the effect of LPS was reduced by treatment with different concentrations of bean hydrolysates. The alcalase hydrolysates produced at 10 and 20 min were not as potent as the ones produced after 80 min. The most potent hydrolysates on NO inhibition were the ones produced at 180 min of hydrolysis showing an IC_{50} of 1.28 ± 0.75 and 1.50 ± 0.31 μM for N bean hydrolysate and P bean hydrolysate, respectively. Expression of iNOS was also measured using Western blot and it was significantly reduced when the cells were treated with the hydrolysates. The hydrolysates also affected the expression of COX-2 and the production of PGE_2. Both common bean hydrolysates at the highest concentration potently inhibited production of PGE_2 but expression of COX-2 was highly inhibited at 80 and 120 min for P while for N was at 120 and 180 min. The results suggest that compounds in bean hydrolysates may act not only at the protein expression level but also inhibited the activity of the expressed enzymes.

The activation of NF-κB was also determined because this transcriptional factor controls the expression of pro-inflammatory genes as it was mentioned before. Treatment of common bean hydrolysates (50 and 200 μM) resulted in a significant decrease of NF-κB activation in LPS-induced RAW 264.7 macrophages as measured by firefly luciferase for both concentrations. Moreover, the effect of common bean hydrolysates at 120 min of hydrolysis on nuclear translocation of p50 and p65 NF-κB sub-units was measured. The translocation of p50 subunit was significantly reduced by N hydrolysate at 50 μM. Treatment of P at 50 μM showed a decrease in p50 nuclear translocation statistically different than the positive control ($p < 0.05$). The nuclear translocation of p65 NF-κB subunit was potently inhibited by N hydrolysate at 50 μM. NF-κB/REL family of transcription factors has an important role in coordinating the expression of a wide variety of genes that control immune responses. It was shown that common bean hydrolysates were able to reduce the activation of the primary regulator of the inflammatory process, NF-κB. The inhibition of the release of NF-κB dimer p65 decreased its translocation to the nucleus and consequently decreased gene transcription.

Bioprocessing of Bioactive Peptides

Bioactive peptides are produced naturally from dietary proteins during the gastrointestinal transit. However, there is a need for technological and industrial processes that can be applied to scale-up the production of bioactive peptides.

An alternative is to use by-products of food protein processing companies resulting in a reduction in production cost with the added advantage of an efficient waste disposal. The proteases used for the production of bioactive peptides are obtainable from plants, microorganisms and animals. The number of bioactive peptides obtainable from food proteins is limitless considering the fact that the number of bioactive peptides produced from a single food protein can be increased by optimizing factors such as the type of enzyme, the microorganism used or by serially combining *in vitro* enzymatic hydrolysis with microbial fermentation (*67*).

Conclusion

Recent studies have shown that protein alcalase hydrolysates of two varieties of *P. vulgaris*, showed the most potent inhibition of pro-inflammatory markers after 120 min of hydrolysis while N after 180 min, in agreement with the degree of hydrolysis. Common bean alcalase hydrolysates of both varieties, after 120 min of hydrolysis, inhibited inflammation through modulation of NF-κB pathways, blocking translocation of p65 subunit. It is concluded that the consumption of partially hydrolysed beans may provide valuable functional ingredients to protect against chronic inflammation.

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Chapter 15

Nutritional and Bioactive Compounds of Bean: Benefits to Human Health

**Hércia Stampini Duarte Martino,*,1 Solange Mara Bigonha,[1]
Leandro de Morais Cardoso,[1] Carla de Oliveira Barbosa Rosa,[1]
Neuza Maria Brunoro Costa,[2] Lucía de Los Ángeles Ramírez
Cárdenas,[3] and Sônia Machado Rocha Ribeiro[1]**

**[1]Nutrition and Health Department, Universidade Federal de Viçosa,
PH Rolfs Avenue, s/n, Viçosa, Minas Gerais, 36570-000, Brazil
[2]Pharmacy and Nutrition Department, Universidade Federal do Espírito
Santo, Alto Universitário, s/n, Alegre, Espírito Santo, 29500-000, Brazil
[3]Agriculture, Foods and Nutrition College, Universidad San Francisco de
Quito, Av. Diego de Robles y Vía Interoceánica, Cumbayá, Ecuador
*E-mail: hercia@ufv.br**

The bean (*Phaseolus vulgaris* L.) is an important legume used for human nutrition. It is the major source of protein, dietary fiber, minerals and vitamins for many individuals and has potential to meet 10-20% of the recommended daily amount of certain nutrients for adults. The chemical composition of bean cultivars varies widely at 15-30% protein, 60-70% carbohydrates, and 0.7 to 2% lipids. The bean carbohydrates are composed primarily of starch, following by dietary fiber and α-galactosyl derivatives of sucrose. The major proteins of bean are globulins (54-79%) and albumins (12-30%) and the presence of protease inhibitors (α-amylase, chymotrypsin and trypsin), lectins and lipoxygenase have been verified. Furthermore, it has flavonols, isoflavones, phenolic acids, tannins and phytic acid. The processing, especially soaking and cooking may reduce the content phytochemicals with effect antinutritional, increase the minerals bioavailability and protein quality. Several studies have demonstrated the functional effects of compounds present in the bean on glycemic control, protection against oxidative stress, improvement in serum lipid profile, antihypertensive effects, chemopreventive effects,

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effects on obesity and metabolic syndrome. This chapter aims to support the discussion on chemical composition of raw and processed beans, and the impact of its chemical components on human health.

Introduction

History of Beans in Human Nutrition

The common bean (*Phaseolus vulgaris* L.) is an important legume used for human nutrition. It is among the oldest foods, dating back to the earliest records of human history. Archaeological findings indicate the existence of domesticated beans about 10,000 years B.C. In fact, the ruins of ancient Troy provide evidence that beans were the food staple preferred by Trojan warriors (*1*).

Most historians attribute the spread of beans throughout the world to war, since the food was the fundamental dietary component for many warriors. Naturally, the great explorers helped expand the use and cultivation of beans to the most remote regions of the planet. Currently, this legume is widely consumed in Mexico, Central America, South America and African countries (*1*).

Beans are one of the most economically and socially significant agricultural products in Brazil, and they figure as a characteristic food in the Brazilian diet. Seven out of every ten Brazilians eat beans daily. Brazil is the world's largest producer of beans, and 3.6 tons of beans were collected in the harvest of 2011/2012. Furthermore, Brazil stands out as the world's largest consumer, accounting for 20.4% of total consumption worldwide (*1*). Other legumes, such as lentils and soybeans, represent less than 1% of total legume consumption (*2*), confirming the Brazilian preference for beans.

The high consumption rate of beans in Brazil is due in large part to the social, economic, and cultural background of the foodstuff as well as its traditional importance in the dietary habits of the Brazilian people (*3*). The average daily consumption of cooked beans in Brazil is 183.0 g/day with 177.9 g/day the average consumption among the urban population and 208.1 g/day the average consumption among the rural population (*4*).

The genus *Phaseolus* includes all known species of bean with *Phaseolus vulgaris* L. the most commonly found variety. There are approximately 40 cultivars of beans grown and consumed in Brazil, among which *Carioca*, *Preto*, *Caupi, Roxo* and *Mulatinho* are the most widely distributed (*5*). The *Carioca* cultivar is the most accepted throughout Brazil comprising 52% of the cultivated area of bean in the country. The black bean, grown in 21% of the bean producing area, is more frequently consumed in the states of *Rio Grande do Sul*, *Santa Catarina*, south and east of the *Parana*, *Rio de Janeiro*, southeastern *Minas Gerais* and south of the *Espírito Santo*. Cowpea bean is the most consumed in the North and Northeast, and corresponds to 9.5% of the cultivated area. In the rest of Brazil, the grain has little or no commercial value or acceptance (*6*).

Recently, the commercial cultivation of genetically modified beans (GM) developed by the Brazilian Agricultural Research Corporation (*EMBRAPA*) was approved by the National Technical Commission on Biosafety. GM cultivars are the result of more than 10 years of research carried out by *EMBRAPA* Genetic Resources and Biotechnology (Brasília, DF) and *EMBRAPA* Rice and Beans (*Goiânia*, GO). The new cultivars, called *EMBRAPA* 5.1, have economic and environmental advantages which include greater yields, increased productivity, and minimized need for chemicals which may be detrimental to the environment (*6*).

The genetic breeding programs emphasized increases in plant productivity and resistance to pest attack. However, currently, the breeding methods are being used to improve the nutritional quality of food crops, thus enabling the development of cultivars rich in vitamins and minerals essential to human metabolism.

The development of biofortified beans with minerals is an important example of the evolution of genetic breeding. Moving beyond advances which simply improve efficiency and economy of crop production, new innovations seek to increase the nutritional quality of foods such as increasing iron and zinc content (*7*). This strategy may be an effective alternative to merely increasing the food supply for reducing micronutrient deficiencies in low-income populations, especially in developing countries like Brazil, where the prevalence of iron deficiency anemia is nearly 50% of the population (Jordão et al., 2009). Thus, the daily use of these cultivars for human consumption, as part of a balanced diet, can help improve health by preventing or minimizing various chronic diseases (*8*).

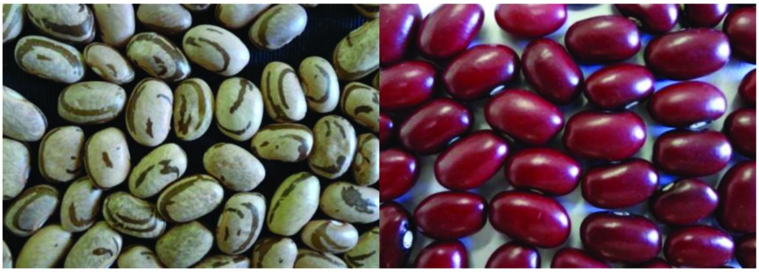
This chapter aims to support the discussion on chemical composition of raw and processed beans, and the impact of its chemical components on human health.

Chemical Composition and Nutritional Value of Bean

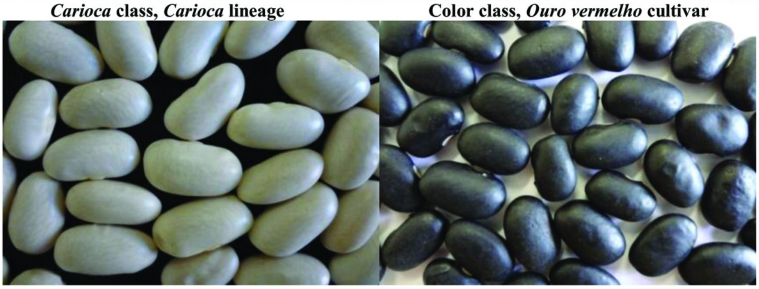
The bean generally consists of the tegument, which comprises approximately 9% dry matter, the cotyledons, 90%, and the embryonic axis, 1% (*9*). The bean cultivars may have different skin and coat colors (white, yellow, black, dark brown, red, green, bluish gray, among others) which can be used for the classification of grains (Figure 1). Currently in Brazil, the beans are classified into classes: white (subclasses: *brancão*, white, among others), black, color (subclasses: cranberry, yellow, mulatto, *canapu*, evergreen, among others), *carioca*, and mixed (*10*).

Just as with the physical characteristics, the chemical composition of bean cultivars varies widely. Certain bean cultivars grown in Brazil have been observed to possess 15-30% protein, 60-70% total carbohydrate, and 0.7 to 2% lipids. This composition varies depending on the cultivar, growing season, geography location and environmental stress (*11–13*) (Table I).

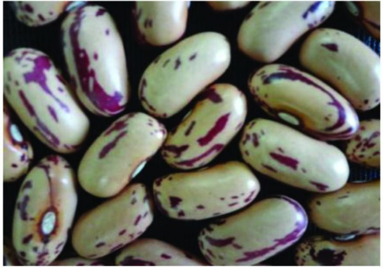
This legume is the major source of protein, minerals, vitamins and dietary fiber for many individuals due to its low cost. It has the potential to meet 10-20% of the recommended daily amount of certain nutrients for adults. The bean is consumed by all social classes, especially by those of low socio-economic status for whom the bean provides a substantial portion of daily nutrition.



Carioca class, *Carioca* lineage
Color class, *Ouro vermelho* cultivar



White class, *Ouro Branco* cultivar
Black class, *Ouro Negro* cultivar



Color class, *Carnaval-MG* cultivar

Figure 1. Physical characteristics of bean cultivars (Phaseolus vulgaris L.) grown in Brazil.

The lipid content in beans is very low, ranging from 0.8 to 2.32% (*15*, *18*). Neutral lipids (triglycerides, free fatty acids, sterols and sterol esters) are the predominant class (30 to 40%) of total lipids. Bean presents a highly variable fatty acid profile that generally includes a substantial amount of unsaturated fatty acids (*19*). In some cultivars, unsaturated fatty acids represent 65-87% of total lipids with α-linolenic acid (37-54%), linoleic acid (21-28%) and oleic acid (7-10%) predominating (*20*). Also present are saturated fatty acids such as palmitic acid which correspond to 10-15% of the total lipids (*21*).

Beans are a good source of water-soluble vitamins especially thiamine (0.86 to 1.14 mg/100 g), riboflavin (0.136 to 0.266 mg/100 g), niacin (1.16 to 2.68 mg/100 g), pyridoxine (0.336 to 0.636 mg/100 g) and folic acid (0.171 to 0.579 mg/100 g); however, they are poor sources of fat-soluble vitamins and ascorbic acid (*18*).

Table I. Chemical composition of cultivars of beans (*Phaseolus vulgaris* L.) cultivated in Brazil (g/100g dry bean) (*13–17*)

Compounds	***Cultivars***					
	Vermelho	*Rajado*	*Carioca*	*Preto*	*Roxinho*	*Mulatinho*
Protein	23.2	23.8	23.5	26.1	25.6	20.2
Carbohydrates	74.5	70.7	67.2	70.9	45.7	74.9
Lipids	1.4	1.8	1.5	2.3	2.3	1.0
Ash	3.7	3.7	4.2	4.3	4.9	3.8
Total Dietary Fiber	22.5	25.2	25.8	26.1	21.5	25.2
Soluble Fiber	3.9	4.5	7.9	5.7	4.9	3.7
Insoluble Fiber	18.8	20.7	17.9	24.6	21.7	21.6

Bean Carbohydrates

The carbohydrate content in several cultivars of beans varies from 40% to 70%. Starch is the main carbohydrate and may vary among plant cultivars (*22*). In addition to starch, beans contain non-digestible carbohydrates, among which stand out the dietary fiber (pectin, gum and mucilage, cellulose and hemicellulose) and α-galactosyl derivatives of sucrose (raffinose, stachyose and verbascose). In some cultivars of beans grown in Brazil, dietary fiber content varies from 15 to 39.39% (*15*, *23*). The cooked bean cultivar Carioca, for example, contains an estimated 9.61% cellulose, 0.46% hemicellulose, 1.65% lignin, 0.81% pectin, 5.78% protopectin, and less than 0.5% phenolic substances (*16*).

The content of the α-galactosyl derivatives of sucrose in raw beans also varies greatly between various cultivars. These derivatives have been observed in quantities that range from 0.3 to 14.1 mg of raffinose, 2.7 to 63.0 mg of stachyose, and 0.1 to 38.5 mg of verbascose per 1 g of bean (*24*, *25*). Among the common beans (*Phaseolus vulgaris*, L.), the black and purple varieties contain a lower quantity of α-galactosides. In white beans as well as navy, kidney, lupine and lima beans, raffinose is the main oligosaccharide which exhibits a flatulence-causing effect (*26*).

The absence of the enzyme α-galactosidase (EC. 3.2.1.22), responsible for the cleavage of terminal α-galactosyl moieties, leads to the accumulation of non-digestible oligosaccharides in the lower intestine (*22*). Thus, raffinose, stachyose and verbascose are fermented by intestinal flora, producing gases (carbon dioxide, hydrogen and methane) which, upon accumulation in the intestine, can cause adverse effects such as bloating, flatulence and diarrhea (*27*).

The adverse effects caused by non-digestible oligosaccharides can be minimized through the use of appropriate dietary and food preparation techniques such as soaking, grilling, germination, and treatments with gamma irradiation or galactosidases from exogenous bacteria or plants which can reduce the content of non-digestible oligosaccharides (*28*, *29*). These oligosaccharides can be reduced by 31-43% when legumes are soaked in water for 12 h., 44-50% when beans are cooked (97 °C for 35 min., the proportion of legume-water 1:3 (w/v)), and 47-62% when the two methods are combined (soaking in water and cooking) (*30*).

Bean Proteins

The different cultivars of beans have high protein content (15-35%) that is strongly influenced by gene expression, which modulates the synthesis and accumulation of specific fractions of proteins, and also by environmental factors such as geographic location and season.

The major proteins of bean are globulins (54-79%) and albumins (12-30%). In contrast to other legumes, common beans contain high amounts of glutelin (20-30%) (*31*, *32*). Phaseolin is a 7S trimeric glycoprotein (globulin) that represents about half of the total protein content of the common bean whereas the other globulin fraction (11S) represents only 10% (*31*, *33*). Some varieties of beans can contain up to 11 different variants of phaseolins, which consist of 2-6 subunits (*34*) with specific molecular weight for each subunit ranging from 54.7 to 41.1 kDa. Those with 3 and 4 subunits accounted for nearly 75% of the total (*35*).

In addition, bean contains several protease and α-amylase inhibitors, lectins and enzymes (such as lipoxygenase). The protease inhibitors (Kunitz trypsin, and trypsin inhibitor and chymotrypsin Browman-Birk), constitute only about 2.5% of the total protein content of beans; however, they contribute approximately 40% of the total cysteine in bean proteins (*20*). The content of protease inhibitors in beans is dependent on the genotype, the growing site, and the environmental conditions. In five cultivars of beans grown in Mexico, trypsin inhibitor (TI) content ranged from 6.3 to 14.5 Units of TI/mg of dry beans (*36*). However, in 21 cultivars grown in Brazil, TI content was greater than that observed in the 5 cultivars of Mexico (59.93 to 151.07 Units of TI/mg of dry beans) (*5*). Alonso, et al. (*37*) observed content of 3.97 Unit of chymotrypsin inhibitor/mg of dry grain, in bean *Athropurpurea* cultivar grown in Spain. Proteases inhibitors, as well as other compounds present in beans with active anti-nutritional potential, are sensitive to certain processing techniques and may be reduced to negligible concentrations after cooking in water (*38*).

The amylase inhibitors found in beans are active against α-amylase produced by the larval phase of insects, and thus play an important role in physiological protection against insect attack. These inhibitors are also capable of inhibiting human salivary and pancreatic α-amylases. Due to its specificity, the bean α-amylase inhibitor is considered an antinutrient in human nutrition (*39*). The activity of α-amylase inhibitor is expressed as a percentage of residual α-amylase activity assayed in the presence of the extracts of the different food samples. In different bean cultivars, activities ranging from 39.4% to 89.9% inhibition have been reported (*40*)

Lectins can interfere in the metabolism of animals and man. The lectins have the ability to bind to specific receptors in the intestinal epithelium; this interaction interferes in the absorption and utilization of nutrients, causing poor performance in developing animals (*41*). The content of lectin in beans varies from 6 to 12% of their total proteins (*42*), and, in some cultivars, may be 2-3 times higher than observed in soybean (*36*). It has been shown that the concentration of the bean lectin can be influenced by the region of cultivation, and bean cultivar was found to contain lectin ranging from 0.42 to 8.89 hemagglutinin activity units/g bean (*36*, *43*). In general, most of the lectin content is almost 100% inactivated during domestic heating processes (*38*). Other treatments, such as extrusion and autoclaving, are also effective in reducing the hemagglutinating activity (*37*, *44*). Significantly, microwave heating does not eliminate the hemagglutinating activity beans that were cooked until release a cooked smell and there were no toasted grain (*45*).

It has been reported that beans contain two isoenzymes of lipoxygenase, identified as A and B. Both proteins have a molecular weight of 100,000, contain 1 atom of iron, and appear to be composed of a single peptide chain (*46*). They catalyze the oxidation of polyunsaturated fatty acids such as linoleic (18:2) and α-linolenic (18:3) to produce unsaturated fatty acid hydroperoxides (*47*). Despite the potential to alter the nutritional value of beans, the activity of lipoxygenase can be reduced to residual levels (16%) by boiling the beans in water for 15 min. at 60 °C or 5 min. at 70 °C (*48*).

Other Components of the Beans

Beans contain other compounds such as flavonols, isoflavones, phenolic acids, tannins and phytic acid. The flavonols quercetin and kaempferol are the most abundant flavonoids in foods. It has been reported that quercetin content ranges from 0 to 24.2 mg/g of dry beans and kaempferol content ranges from 0 to 209.4 mg/g of dry beans (*25*, *40*) (Table II). However, after cooking the beans, this component may be reduced by 43% to 47% for quercetin and kaempferol, respectively (*25*, *49*).

Isoflavones are a subclass of a large group called flavonoids the consumption of which due to its modulation of estrogen activity (*50*), has been related to a reduced risk of cardiovascular disease and cancer development, particularly for breast and prostate cancers (*51*, *52*). In raw beans, the major isoflavone is genistein (0 to 129.1 mg/g) followed by daidzein (0 to 9.7 mg/g) (*25*, *40*) (Table II). In addition to these isoflavones, the presence of coumestrol (2.6 to 9.7 mg/g), a phytochemical that also affects estrogenic processes, has been observed (*25*).

The phenolic acids present in foods, including beans, can be divided into two classes: derivatives of benzoic acid (*p*-hydroxybenzoic, vanillic and gallic acid) and derivatives of cinnamic acid (ferulic acid, *p*-coumaric acid, and caffeic acid). In beans, the presence of *p*-hydroxybenzoic acid (5.7 to 13.8 μg/g), vanillic acid (5.2 to 16.6 μg/g), *p*-coumaric acid (3.2 to 6.8 μg/g) and ferulic acid (17.0 to 36.0 μg/g) has been verified (*25*).

Table II. Phenolic acid and isoflavone composition of raw beans (*Phaseolus vulgaris* L.) grown in Italy and Mexico (*25*, *40*)

Cultivars	***Flavonols (µg/g)***		***Isoflavones (µg/g)***	
	Kaempferol	*Quercetin*	*Daidzein*	*Genistein*
Badda bianco FRM	11.4	u.d.t.	20.2	3.24
Badda bianco SG	38.1	u.d.t.	6.5	2.16
Monaco Mussu Niuru	38.1	24.1	101.0	u.d.t.
Poverello di Rotonda AF	57.2	24.2	13.5	21.6
Tabacchino	61.0	17.7	33.8	u.d.t.
Bianco di Rotonda TL	u.d.t.	u.d.t.	37.0	0.72
G-12906	10.5	13.3	23.8	5.6
Notom	12.4	20.0	25.8	6.4
FJMar	39.6	27.9	43.6	4.2

u.d.t.: under detection threshold.

Tannins are compounds found in most plants and roots that may be present in the wood, bark, leaves, fruits, seeds, and sap. In beans, tannins are located primarily in the hulls, and in small amounts in the cotyledons (Santos, 2000). These compounds are present in quantities ranging from 0.03 to 38.1 mg of catechin equivalent/g of dry beans (*15*, *30*, *36*). This content is associated with the color of the beans and the species (*53*). Tannins are hydrophilic, thermolabile compounds; thus, baked beans in water (97 °C, 35 min) results in a 30% reduction of tannin content, and soaking the beans in water for 2 h. before cooking (97 °C, 35 min) results in a 70% reduction (*15*, *30*) (Table III).

The phytic acid, also known as phytate, inositol hexaphosphate (IP^6), myo-inositol, myo-inositol and inositol phosphate, is the main form of phosphorus reserve in bean which is essential for germination of the plant. In the raw beans, the phytic acid content can vary from 7.8 to 27.1 mg/g (*15*, *25*, *30*, *55*). Of that, 81-89% corresponded to the IP^6 form (*15*), and 26-53% is located on the tegument (*55*). Only IP^6 and IP^5 exert a negative effect on the bioavailability of minerals. The other compounds formed such as IP^4 and IP^3 have low capacity to bind minerals or the complexes formed by binding are more soluble. Furthermore, these phytic acid compounds may have antioxidant properties (*56*–*58*).

Legumes are rich in saponins which are known to provide many health benefits such as hypocholesterolemic and anticarcinogenic properties, and may stimulate the immune system. However, they are toxic at high concentrations and may affect nutrient absorption either by enzyme inhibition during digestion or by the interaction with zinc (*59*). The main saponin components in legumes are the group B saponins. The group B saponins have the aglycone, sapogenol B, as sapogenin, which make up a majority of the saponins in legumes (*40*). Information on the saponin content in beans is scarce and contradictory. Some authors have reported the presence of small amounts and traces of saponins in bean varieties (*60*, *61*) while others observe the presence of a high content of these compounds (7.62 mg/g) (*62*). Bean saponins are sensitive to heat and cooking medium. Reduction of total content was observed after soaking in water (12 h) followed by cooking in water at (100 °C, 35 min) (82-90%), autoclaving for 15 min (100%), and cooking in water at (100 °C, 35 min) (52-68%) (*30*).

Table III. Content and reduced levels of tannins on a dry basis, for the bean cultivars (*Phaseolus vulgaris* L.) grown in Brazil and Turkey (*15*, *54*)

Cultivars	***Catechin equivalent/g dry bean***	***Reduction after cooking (%)***		
		Cooking with steepwater [1]	*Cooking without the steepwater* [2]	*Cooking without soaking* [3]
Ouro Branco	0.03	74	76	68
Diamante Negro	0.61	69	71	64
BRS Radiante	1.82	81	82	80
Pérola	1.02	82	83	80
Talismã	0.94	82	83	81
Dermason	0.64	82	nd	73
Horoz	0.57	82	nd	73
Çali	0.72	81	nd	74

nd not determined; [1] beans soaking for 15 h and baked in steepwater using domestic pressure cooker (40 min.); [2] beans subjected to the soaking and baking as described above but without the steepwater; [3] beans baked without soaking under the same cooking conditions mentioned above.

During processing, storage, fermentation, germination and digestion of beans, IP^6 may be dephosphorylated to produce compounds such as pentaphosphate (IP^5), tetraphosphate (IP^4), triphosphate (IP^3), and possibly inositol diphosphate (IP^2) and monophosphate (IP^1), by the action of endogenous phytases (*15*, *30*, *56*, *63*). The content of phytic acid in the beans can be reduced by processing (*15*), and reduction of phytic acid components may be observed after soaking the beans for 12 h to 19%, and 65% after soaking in water (2 h) followed by cooking (97 °C, 35 min) (*30*) (Table IV).

Table IV. Effect of processing methods on the levels of phytic acid of three bean cultivars (*Phaseolus vulgaris* L.) (mg/g dry bean) (*30*). Adapted with permission from reference (*30*). Copyright 2006 Elsevier

Treatment	*Awash cultivar*		*Beshbesh cultivar*		*Roba cultivar*	
	Phytic acid	*Reduction (%)*	*Phytic acid*	*Reduction (%)*	*Phytic acid*	*Reduction (%)*
Unprocessed seeds (control)	23.5		24.7		17.3	
Water soaking (12 h in plain water)	19.3	18	20 .0	17	14.1	19
Sprouting for 24 h	6.3	73	15.7	35	12.5	28
Cooking of un soaked seeds	17.6	25	17.3	28	12.8	26
Water soaking + cooking	8.2	65	8.7	64	6.7	61
Sodium bicarbonate soaking + cooking	8.5	64	8.9	63	6.8	61
Autoclaving of un soaked seeds	8.2	65	8.4	65	6.9	60
Soaking(H2O) + autoclaving	8.0	66	8.4	65	6.6	62
Sprouting for 24 h + autoclaving	0.6	98	1.3	95	0.4	98
Sprouting for 48 h + autoclaving	nd	100	nd	100	nd	100

Protein Quality

The qualitative and quantitative composition of essential or indispensable amino acids and protein digestibility are basic parameters for evaluating the quality. According to Blanco and Bressani (*64*), the protein quality refers to its ability to meet the nutritional requirements of man for essential amino acids, and nitrogen is not essential for protein synthesis.

The bean proteins contain all essential amino acids, is rich in lysine. However, it contains limited amounts of sulfur amino acids (cysteine and methionine) (*13*, *65*, *66*) (Table V). Thus, the combination of beans and cereals (which are rich in sulfur amino acids) is needed to meet human nutritional needs. An alternative may be the development of new bean cultivars that meet the recommendations for essential amino acids.

Table V. Amino acid composition for the cultivars of beans (*Phaseolus vulgaris* L.) grown in Brazil, cooked with the soaking water (g/100g wet matter) (*15*, *70*)

Amino acids	*Cooked Cultivars*				*Raw Cultivars*	
	Ouro Branco [1]	*Diamante Negro* [1]	*BRS Radiante* [1]	*Talismã* [1]	*Peróla* [2]	*Carioca* [2]
	Indispensable					
Phenylalanine + Tyrosine	1.19	1.57	1.42	1.62	1.29	0.94
Histidine	0.39	0.36	0.37	0.44	0.58	0.44
Isoleucine	0.43	0.55	0.56	0.62	1.16	0.81
Leucine	0.84	1.15	1.16	1.20	1.77	1.30
Lysine	0.67	0.97	1.05	0.94	1.78	1.25
Metionine	0.20	0.24	0.22	0.27	0.26 [3]	0.17 [3]
Cysteine	nd	nd	nd	nd	-	-
Threonine	0.48	0.59	0.51	0.53	0.98	0.75
Tryptophan	nd	nd	Nd	nd	nd	nd.
Valine	0.56	0.64	0.64	0.72	1.30	0.91
Total indispensable	4.76	6.07	5.93	6.34	9.12	6.58
	Dispensable					
Alanine	0.61	0.67	0.64	0.69	0.90	0.70
Arginine	0.80	0.79	0.7	0.93	2.06	1.60
Aspartic acid	1.70	1.75	1.66	1.78	3.80	2.77
Glutamic acid	2.15	2.12	1.99	2.26	4.29	3.15
Glycine	0.49	0.51	0.47	0.51	0.81	0.59
Proline	0.62	0.65	0.63	0.72	0.90	0.70
Serine	0.76	0.84	0.74	0.82	1.35	1.03
Total dispensable	7.13	7.33	6.83	7.71	14.11	10.55
Total of amino acids	11.89	13.40	12.76	14.05	23.22	17.13

[1] Cooked bean; [2] raw bean; [3] metionine + cysteine; nd: not determined.

The method of protein digestibility-corrected amino acid score (PDCAAS) is recommended as a standard method for evaluating the quality of food proteins. The quality of the protein is based on essential amino limiting in which values above 1 indicate that the protein is of good quality, containing the amino acid essences able to meet the needs of the human diet (*13*). In studies that evaluated different cultivars of Brazilian beans, cooked beans had an intermediate PDCAAS (0.50-0.62) (*13*, *15*) (Table V). However, it is noteworthy that in these studies the values of PDCAAS may be underestimated because only methionine was quantified, and the pattern of the FAO considers the value of 25 mg/g protein to be methionine + cysteine.

The nutritional quality of bean proteins is influenced by the species, variety botany, concentration of compounds with active anti-nutritionals, storage time, heat treatment and, in general, is lower than that of animal protein (*67–69*). The raw bean has reduced protein digestibility which is associated with the action of compounds with active anti-nutritional substances present in the bark (tannins) and cotyledons (proteins, tannins, phytates) (*69*). *In vitro* protein digestibility ranged from 18.03% to 48.32% in 21 cultivars grown in Brazil (*5*).

The nutritional quality of raw bean protein can be increased by processing, especially by wet heating (*14*, *30*). In *in vitro* studies, the maceration to 12 h, following cooking or 1 h under normal pressure increased the protein digestibility, on average, from 43.3% to 63.7%, in four bean cultivars grown in Brazil (*14*), and 65.63 to 73.53; 71.14 to 78.88 and 80.66 to 90.31 in three cultivars grown in East Africa. *In vivo* studies, the relative efficient protein ratio and true digestibility in eight varieties of beans grown in Brazil, boiled in water, ranged from an average of 30.92% to 60.82% and 67.2% to 93.97%, respectively (*22*, *67*). The improvement of *in vitro* protein digestibility of beans may be attributed not only to the removal/reduction of antinutrients, but also to the structural disintegration of the native protein, including enzyme inhibitors and lectins, differential solubility of the individual oligosaccharides and their diffusion rates, phytase activity to break down phytic acid in the seeds and the development of endogenous α-galactosidase activity to diminish oligosaccharides (*30*).

Mineral Bioavailability

Six bean cultivars grown in Brazil presented relevant levels of some minerals such as calcium, iron and zinc, for which intake is marginal for some population groups in social vulnerability (Table VI). It appears that the contents of some minerals, especially antioxidants, have not yet been quantified. The mineral content of beans may be reduced during processing, as soaking alone, and soaking followed by cooking (*71*).

Genetic breeding has been used to improve the quantity and quality of bean's minerals. Biofortified beans seem to be a promising vehicle for increasing intakes of bioavailable iron, especially in human populations that consume these beans as a dietary staple (*72*). Some cultivars of bean of the germplasm collection of the International Center for Tropical Agriculture in Colombia have enough genetic variability to increase the content of iron and zinc in 80 and 50%, respectively. The use of hydroponics, for example, can increase the content of iron and zinc to 98 and 65%, respectively, which leads to a reduction of reasons phytate/phytate and iron/zinc in 38% and 46%, respectively (*73*).

Table VI. Mineral composition of cultivars of raw beans (*Phaseolus vulgaris* L.) grown in Brazil (mg/100g dry matter) (*5*, *15*, *21*)

Cultivars	***Minerals***						
	Fe	***Ca***	***Mn***	***Mg***	***Cu***	***Zn***	***K***
Vermelho	5.6	137.5	2.1	198.6	1.2	3.4	1492.5
Rajado	8.8	60.0	1.6	190.0	1.2	4.2	1510.0
Carioca	5.3	172.2	1.6	205.9	1.8	3.2	1332.5
Preto	5.4	174.2	1.9	214.1	2.7	3.2	1512.5
Roxinho	5.4	172.5	1.5	216.0	2.3	2.9	1362.5
Mulatinho	9.6	60.0	1.0	210.0	0.9	3.0	1200.5

An increase in a mineral’s concentration, alone, cannot reflect a proportional increase in the content of mineral absorbed since the minerals’ bioavailability is also modulated by the concentration of inhibitor compounds or absorption enhancers. A comparative study with red beans including Andean varieties, conventional (49 µg Fe/g ; “Low Fe”) and biofortified (71 µg Fe/g; “High Fe”), using an animal model, revealed hemoglobin maintenance efficiencies and liver ferritin values higher in the group treated with biofortified beans (*72*). However, in a study with women with low iron status that evaluated iron absorption through erythrocyte incorporation of stable iron isotopes, it was found that in spite of similar polyphenols levels and a phytic acid:iron molar ratio, the iron absorption from the biofortified bean was 40% lower than the conventional bean resulting in equal amounts of iron absorbed (*74*).

Bioavailability is also related to the intrinsic characteristics of the food matrix, as bean color, and extrinsic, as the type of processing (dehulled, soaking and cooking). Mineral bioavailability in the beans, especially of the iron, is higher in white beans when compared with colored beans (*75–77*), and it is increased after removal of the hull (*78*). In both cases, the differences in iron bioavailability were not due to molar ratio of iron:phytate, but due to flavonoids in the hulls, especially in the colored hull, which contributed to the low mineral bioavailability.

The soaking alone and soaking followed by cooking preparation methods, contribute to the release of minerals in free form which increases their extractability in HCl of Na, K, Ca, Mg, P, Fe, Mn and Cu (*71*). The HCl extractability is a bioavailability index of minerals, thus soaking in water followed by cooking can be considered beneficial for a technique to improve the mineral bioavailability. In an animal study, it was found that some varieties of beans, when boiled, with or without steepwater, presented zinc bioavailability higher than that of zinc carbonate ($ZnCO_3$, standard diet). However, the cooking methods did not uniformly affect the bioavailability of different varieties, and this changes existing were attributed to the intrinsic characteristics of the raw material, especially the relationship phytate:calcium:zinc. In the same study it the iron bioavailability in the *Ouro Branco* cultivar, after soaking water following by cooking, was similar to the pattern (ferrous sulfate, $FeSO_4$) (*79*).

The data suggest that use of the individual genetic breeding, by increasing the mineral content or reducing compounds that reduce the bioavailability of these minerals, or processing (peeling, soaking, cooking etc.) may not be able to improve the bioavailability of minerals. Thus, it is recommended that different techniques are combined to improve the mineral bioavailability of beans.

Functional Properties of Bean

The beneficial health properties of beans are also well known, and due to the high incidence of chronic diseases related to lifestyle, beans have gained a new status in dietary recommendations. Historically considered a staple food in developing countries to stop hunger, it has been remade as a novel functional food to reduce the risk of illness, such as cardiovascular disease, due to its cholesterol-lowering properties. For instance, Geil and Anderson (*18*) reported a significant reduction in blood lipids in subjects consuming 75 to 200 g of dry kidney beans daily. Baked beans were also reported to reduce total- and LDL-cholesterol levels in hypercholesterolemic rats and pigs fed high-saturated fat diets supplemented with 1% cholesterol (*80*, *81*).

The health potential of Brazilian beans (*Phaseolus vulgaris,* L.) was demonstrated in hypercholesterolemic rats fed black, red and *carioca* (*Pinto* cultivar) beans, whose blood total cholesterol levels were reduced by 16%, 12% and 11%, respectively (*82*). The skinned black beans promoted 35% reduction on the blood levels of total cholesterol, as well as a higher excretion of fecal cholesterol in hypercholesterolemic rats (*82*). Ramírez-Cárdenas (*79*) studied the functional effects of five Brazilian beans cultivars (*Phaseolus vulgaris*, L.) on blood lipids and glucose in adult Wistar hypercholesterolemic rats. The black bean (*Diamante Negro* cultivar) was the most effective in lowering blood cholesterol, and triglycerides and liver lipid deposition. HDL/total cholesterol ratio and fecal lipid excretion were higher in the animals fed black beans compared to the other cultivars and control group (fed high fat, high-sucrose diet). The Pinto bean (*BRS radiante* cultivar) was more effective in reducing blood glucose, whereas the White bean (*Ouro Branco* cultivar) showed no effect on blood lipids and glucose, higher lipid deposition in the liver, and lower fecal excretion of lipids. The other pinto bean cultivars (*Perola* and *Talismã* cultivars) showed mixed results. The functional properties of these bean cultivars were significantly related to their fiber and tannin content, but showed no correlation with phytate and amino acid contents.

The study of Birketvedt, et al. (*83*) with 62 overweight and obese subjects consuming diets supplemented with bean extracts (white kidney beans mixed with locust beans) showed 6% reduction on their blood total cholesterol in short (three months) and long term (12 months) interventions. Winham, et al. (*84*) observed similar effect in 16 individuals with moderate insulin resistance, whose blood total- and LDL-cholesterol levels were reduced by around 8% after 8 weeks of *Pinto* beans consumption.

The mechanisms of action to explain the hypocholesterolemic effects of beans, although not completely elucidated, are associated with the multiple components of the legumes (*85*), such as plant protein, soluble fiber, saponins, plant steroids, polyphenols, and phytate.

The antinutrients found in legumes (protease inhibitors, saponins, lectins, polyphenols, and phytate) are well known for reducing their nutritional value, digestibility and mineral bioavailability (*86*). Many of these compounds and their hydrolysis products, however, may exert health benefits in lowering blood lipids and glucose when fed at low levels in the diet. Polyphenols, phytate, protease inhibitors, saponins, lignans, and plant sterols are also associated with the reducing the risk of many types of cancer (*59*).

The undesirable effects of bean intake, caused by the flatulence derived from the fermentation of α-galactosyl derivatives, such as raffinose, stachyose and verbascose may be one of the responsible factors for avoiding beans as part of the diet for many people. Food processing to reduce flatulence in legumes and/or to improve the bioactive components is a promising strategy to increase the healthy benefits of bean consumption and, thereafter, the functional food marketing. On that note, some patents have been registered and many others may come out in the future, which certainly will contribute to increase the dietary intake of beans or their components and reduce the risk of cardiovascular disease, diabetes mellitus, obesity, and cancer, when consumed regularly in the context of a healthy diet.

Beans are complex foods with great functional potential, considering that its contain several constituents that present biological effects investigated separately: protein, dietary fiber, saponins, esterols, phenolic compounds, protease inhibitors, α-amylase inhibitors, lectins, α-galactosyl derivatives of sucrose, phytates.

Plant Protein

Several animal and human studies have showed the effects of plant proteins of beans, soybean and other legumes in reducing the levels of blood cholesterol compared with animal proteins (*87*). The majority of these studies have been restricted to the comparison between two well known and easily isolated proteins, namely casein and soybean protein. The mechanisms of action are probably related to the composition and the balance between essential and non-essential amino acids (*88*).

The study of Kritchevsky (*89*) in the 1970's have pointed out the role of the lysine:arginine ratio in the genesis of the atherosclerosis process. The relative abundance of lysine in animal protein may inhibit the arginase activity in the liver, raising the availability of arginine for LDL apoprotein synthesis.

Dietary Fiber

Epidemiological evidences indicate the benefit effects of dietary fiber in reducing the risk of CHD, due to its ability to reduce blood lipid concentration, blood pressure, and body weight and the improvement of glucose tolerance, among others (*90*, *91*). The beans are good source of soluble and insoluble dietary fiber, providing approximately 20 to 30 g/100 g in dry matter (*15*). The applicability of beans in food industry, however, is incipient, although their use as source of non-fermentable fiber has been cited (*92*).

The benefits of dietary fiber have been demonstrated in animal studies, as follows: a) dietary fiber promotes significant reductions on total serum cholesterol; b) insoluble fiber (cellulose, and some hemicelluloses) have little effect on serum total cholesterol; c) soluble fiber (pectin, gum, mucilage and some hemicelluloses) exert significant hypocholesterolemic effect; d) reductions on serum cholesterol may be or may be not followed by cholesterol reduction in the liver, aorta and other tissues; e) the major cholesterol-lowering effect is observed in LDL-cholesterol; f) HDL-cholesterol may be reduced, increase or have no effect. Based on these reports, some products have been developed and patented using guar gum and other gel-forming fibers for reducing serum cholesterol (*93*).

The high viscosity of soluble fiber in the gut promotes lower postprandial glucose and lipids (*94*), and is more effective than the insoluble fiber in reducing total and LDL-cholesterol concentration (*94*). The mechanism of the hypocholesterolemic effect of soluble fiber may be partially explained by its ability to absorb bile salts (*95*), which in turn is made unavailable for reabsorption. As a consequence, the cholesterol synthesized in the liver is channeled to the bile acid synthesis. Furthermore, the unavailability of bile acids for micelle formation in the gut reduces cholesterol absorption (*96*). The volume of fecal excretion is increased by soluble fiber intake (*94*, *95*), and also the bile acids (*97*), sterols and lipids (*38*) in the feces. The slow gastric emptying and the high viscosity in the lumen reduce the access of the digestive enzymes and hence, the lipid digestion and absorption (*97*, *98*), improving the satiety. The precise mechanism by which soluble fiber complexes with bile acids is not completely elucidated, although the hydrophobic (phenol groups) and ionic (uronic acids) interactions have been proposed (*98*). The association of soluble fiber to non-digestible cyclodextrins and sterols has been proposed to increase the bile acid binding capacity and hence reduce the levels of cholesterol and LDL-cholesterol (*99*).

Soluble fiber is fermented by the microbiota in the colon producing short chain fatty acids (SCFA), especially acetate, propionate and butyrate. The SCFA are absorbed in the gut and transported through the portal vein to the liver, where the propionate may inhibit cholesterol synthesis, as shown by the study of in rats fed oat bran (*100*).

Several studies indicate the benefits of soluble fiber to reduce blood cholesterol and its potential in dietary manipulation of hypercholesterolemia. While a low-fat diet lowers the concentration of serum cholesterol to about 5 to 10%, the addition of soluble fiber rich foods, including beans, may promote 20 to 30% reduction (*82*, *100*). The magnitude of this effect is considerably important considering that 1% reduction on total cholesterol reduces the risk of CHD to about 2% (*101*).

High-fiber diets are also recommended to dietary manipulation of diabetes, due to their abilities to reduce fasting and post-absorptive levels of blood glucose. The mechanisms of action are not completely elucidated, although, it seems to be related to the mechanical effect of insoluble dietary fiber in the gut, which reduces the intestinal transit time. Conversely, the high viscosity of soluble fiber slows down the gastric emptying, and glucose digestion and absorption, which reduces its concentration in the blood. Soluble fiber also improves insulin sensitivity (*102*), since the SCFA, such as acetate, provide an alternative insulin-independent source of energy capable to replace glucose (*97*). Butyrate also plays an important role in diabetes control, by improving the levels of blood and urine glucose and the urine volume in chemically-induced diabetic rats (*103*).

The addition of 98 to 145 g of dry beans/day is effective to improve metabolic control and exert long term beneficial effects in diabetics. The HCF (High Carbohydrate-High Fiber) program recommends the daily intake of half cup of cooked beans as source of dietary fiber in a meal planned for diabetics (*18*).

Saponins

Based on experimental studies, the saponins show hypocholesterolemic and anticarcinogenic properties, and may stimulate the immune system. The mechanism proposed for the anticarcinogenic effect includes their antioxidant property, the selective cytotoxicity against cancer cells, the modulation of the immune system, and regulation of cells (*104*). Kidney beans (*Phaseolus vulgaris* L.) and other legumes contain saponins conjugated with DDMP (2, 3-dihydro-2,5,dihydroxy-6-methyl-4H-pyrano-4-one). These saponins are supposed to have anticarcinogenic effects and present similar activity to SOD (superoxide dismutase) in removing reactive oxygen species (ROS) (*20*).

Saponins are able to form insoluble complex with β-hydroxy steroids, which reduces cholesterol absorption in the intestine, and increases the fecal sterol excretion. The adsorption of bile acids to dietary fiber is enhanced in the presence of saponins, forming mixed micelles of high molecular weight, which reduces bile acid absorption and increases bile acid synthesis from cholesterol in the liver. Saponin interaction with the mucosal cells increases cell permeability and hence the normal cell function, which increases cell exfoliation and proliferation. The increased cell loss leads to additional fecal cholesterol excretion (*20*).

The hypocholesterolemic effect of saponins was observed in rats fed beans and bean starch (*105*). The addition of 0.3% bean sapogenin to the rat diets for 3 days promoted an increase in the bile cholesterol to about 300%. This study showed that the effect of beans (*Phaseolus vulgaris*, L.) on serum and bile cholesterol, phospholipids, and liver cholesterol esters were dependent on the bean starch fraction, presumably due to the content of sapogenin. The study suggested that the increased bile cholesterol excretion may induce to a significant hypocholesterolemic effect of dietary manipulation.

Plant Sterols

Clinical and epidemiological studies demonstrated that the addition of plant sterols to the diet reduced plasma total cholesterol and LDL-cholesterol levels (*106*). The intake of 3 g/day may reduce cholesterol levels in humans, although at these levels, the concentration of HDL-cholesterol and triglycerides did not change (*107–109*). The proposed mechanisms are associated to the reduced cholesterol solubility in the lumen, the competition with cholesterol to the mucosal sites for absorption and the interference in the uptake of cholesterol by the chylomicrons.

Phenolic Compounds

Nagem, et al. (*110*) reported that quercitrin, isoquercitrin, formonometin, biochanin A, rutin and quercetin were the most effective flavonoids in reducing serum lipids in the rat. In another study it was found that beans cultivated in China showed broad composition of phenolic compounds (catechin, epicatechin, p-coumaric acid, ferulic acid, vitexin, isovitexin, sinapic acid, quercetin) with antioxidant capacity and antidiabetic potential (*111*).

Isoflavones are phenolic compounds in the flavonoid group, which are present exclusively in legumes, particularly the species *Vicia* and *Phaseolus*. Isoflavones are potentially anticarcinogenic. The first studies focused on their ability to reduce the risk of breast cancer, although such effect may be not related to hormonal mechanisms (*112*).

The majority of the studies have focused on the anticarcinogenic effect of soy genistein, due to its antioxidant and tyrosine kinase inhibitor properties (*113*). According to Garrido G, de la Maza C and Valladares B (*112*), precaution is necessary to interpret the actual evidences, since most of the inhibitory effect on tumor growth is obtained at high doses of plant estrogen (10-50 μM), which are much higher than the normal dietary intake. Therefore, no conclusion can be taken from these human protocols concerning the use of soybean and derivatives on primary and secondary cancer prevention.

The isoflavone chemical structure is similar to the steroid hormones, and hence presents estrogen activity. Pharmacological doses of estrogen induced the synthesis of liver lipoprotein receptors in rats and increased the uptake of plasma VLDL and LDL. Possibly the isoflavones show similar effect in reducing the levels of plasma lipoproteins (*88*).

Several clinical studies indicate the ability of the isoflavones to reduce the susceptibility of lipids to oxidation (*114*) and it has been observed that they may present similar effect to digitalin in coronary artery relaxation (*112*).

Tannins also have been reported to show hypocholesterolemic effect in rats fed high-cholesterol diet. Tebib, et al. (*115*) reported that the addition of 1% cholesterol in the rat diet raised the levels of LDL and lowered HDL-cholesterol, but these changes were prevented by the addition of 2% polymeric tannin to the diet.

The benefit effects of polyphenols have counteracted their antinutrient property (*116*). Tannins and other plant polyphenols (anthocyanins and flavonoids) have been considered as protective factors against the effect of free radicals in diseases such as cancer and atherosclerosis (*33*). The inhibition of lipid peroxidation was investigated in an in vitro study with anthocyanins isolated from *Phaseolus vulgaris*. The results show a strong antioxidant activity in liposome systems, reducing malondialdehyde formation induced by UV irradiation (*117*).

Protease Inhibitors

Despite their adverse effect on human health, protease inhibitors may also present benefits, as reported in the literature regarding their anticarcinogenic properties. For instance, brain tumors induced by nitrous compounds in rats were inhibited by purified bean protease inhibitors (*118*). Studies carried out in animals, *in vitro*, and culture cells, as well as epidemiological data have shown low mortality rates due to cancer at high doses of protease inhibitors (*119*). The mechanisms involved in anticarcinogenic effect of protease inhibitors of beans have not been elucidated. Study reports that these compounds isolated of bean affect differentially cell proliferation and cell survival of normal and transformed cells (*120*). The affects cell adhesion patterns of poorly adherent transformed fibroblasts. Both effects, cytotoxicity and increased cell adhesion, represent a new alternative in the use of proteins to diminish or prevent the development of malignancy.

α-Amylase Inhibitors

The nutritional importance of the α-amylase inhibitors is controversial. They certainly reduce the rate of intestinal starch digestion in rats and humans. Rats fed experimentally with α-amylase inhibitors presented some starch in their feces, which suggests that these inhibitors are active *in vivo*, and may reduce the animal growth (*121*). Besides the negative nutritional effect of α-amylase inhibitors, they promote a slower starch digestion and glucose absorption, which contributes to their hypoglycemic effect in rats and humans (*59*). In an *in vivo* study, prolonged administration of an inhibitor of α-amylase isolated and purified from an extract of white kidney beans reduced levels of blood glucose, food intake and body weight gain in rats (*122*).

Consumption of the α-amylase inhibitor causes marginal intraluminal α-amylase activity facilitated by the inhibitor's appropriate structural, physico-chemical and functional properties. As a result there is decreased postprandial plasma hyperglycemia and insulin levels, increased resistance of starch to digestion and increased activity of colorectal bacteria. The efficacy and safety of the amylase inhibitor extracts, however, depend on the processing and extraction techniques used. The extracts are potential ingredients in foods for increased carbohydrate tolerance in diabetics, decreased energy intake for reducing obesity and for increased resistant starch (*123*).

Lectin (Phytohemagglutinin)

Due to its particular carbohydrate-binding property, lectin has wide application in identification of blood types, membrane receptors and malignant cell detection. One of the most interesting applications of lectin in medical research is to prevent rejections in bone marrow transplantations, due to the fact that soy lectins may be used to remove T cells responsible for such rejections. It was also observed that the administration of lectin from soybean and kidney beans slow down tumor growth in rats after the transplant (*124*).

In an *in vivo* study with Wistar rats, a reduction of circulating insulin levels in rats fed a diet containing beans was verified. This reduction was due to an increase in the level of mRNA for the insulin receptor (327%) and GLUT-4 (185%) in the gastrocnemius muscle, which was attributed to the effect of lectin in the grains (*125*).

α-Galactosyl Derivatives of Sucrose

Hydrogen and carbon dioxide are the major gases derived from bacteria fermentation of the nondigestible carbohydrates. Other gases, such as escatol, sulfur gas, indol, volatile amines, and short chain fatty acids (predominantly acetate, propionate and butyrate) are added to form the characteristic odor. The SCFA are absorbed into the portal vein, and in the liver propionate may inhibit cholesterol synthesis, and consequently reduces the level of blood cholesterol. Besides the negative flatulence effect of the gas formation, the fermentation stimulates peristaltism, which helps to prevent constipation (*42*). Naczk, Amarowicz and Shahidi (*26*) reported no health benefit of the α-galactosides of sucrose, but further investigations are necessary to evaluate their physiological effects.

Phytate

Phytate is usually referred in the literature as a chelator capable to reduce mineral bioavailability in humans and animals. The phytate-iron complex, however, may be beneficial for reducing hydroxyl radicals in the lower intestine. Other positive effects of phytate in human health include the reduced levels of serum glucose and lipids and the risk of breast cancer (*126*).

Benefit effects of low concentration of phytate is reported in the literature, concerning its lipid-lowering effect (*126*). Such capacity is related to the capacity to bind zinc, and hence the Zn/Cu ratio, which, in turn, reduce the levels of serum cholesterol and the risk of CHD (*127*). Phytate may also reduce serum glucose and insulin, and such hypoglycemic effect can reduce liver lipid synthesis (*128*). Phytates reduce starch absorption by a diversity of mechanisms. They inactivate α-amylase by binding to the enzyme itself, make a complex with calcium, which is necessary to stabilize the enzyme, and may bind to the starch, changing its gelatinization degree or accessibility to digestive enzymes. Phytate affect the glucose response also by lowering gastric emptying.

The anticancer property of phytate is associated to different mechanisms of action. For instance, the iron-phytate complex reduces the free radical formation, since iron can catalyze lipid peroxidation and DNA damage. The zinc-phytate may reduce cell proliferation, since zinc is necessary for DNA (*128*). Non-digested starch is fermented in the lower intestine producing SCFA, such as butyrate, which have anticarcinogenic property. The fermentation also lowers the pH, which reduces the formation of carcinogenic compounds, such as bile acids and ammonium, by their unsolubilization and neutralization, respectively.

Studies on nutritional value of beans has been carried out for many years, but are still lacking research on composition in minor compounds, as well as trial studies to verify the potential effect of different beans cultivars to modify chronic disease risk factors and to modulate pathophysiological mechanisms involved.

Conclusion

Common bean is an important crop for human nutrition, especially in developing countries given its composition, provides numerous health benefits, as indicated in the dietary manipulation of various diseases such as heart disease, diabetes mellitus and cancer. Although the mechanisms of action of each component of the beans are not completely understood it is likely that the synergistic actions of bioactive compounds of beans make a food with high functional properties. Among the most widely accepted components with functional properties are protein, soluble fiber, saponins, steroids, polyphenols and phytates. Also the antinutritional factors present in legumes such as protease inhibitors, lectins, saponins, phytates and polyphenols, are well known to reduce its nutritional value, digestibility or decreasing the bioavailability of proteins and minerals. Some nutrients, however, and its hydrolysis products may also have beneficial effects on health when found in small amounts in the diet. Despite the variety of beans grown and consumed in Brazil and the evidence about the functional potential of this legume, there is a need to develop research to further the knowledge on the nutritional value and modulation of risk factors for chronic diseases to ensure food and nutritional security.

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Chapter 16

Brazilian Soybean Products: Functional Properties and Bioactive Compounds

Maria Gabriela Vernaza,*,[1,3] Marcio Schmiele,[1] Luz Maria Paucar-Menacho,[2] Caroline Joy Steel,[1] and Yoon Kil Chang[1]

**[1]Cereal, Roots and Tubers Laboratory, Department of Food Technology,
Faculty of Food Engineering, University of Campinas (UNICAMP),
P.O. Box 6121, 13083-862, Campinas - SP, Brazil
[2]Departamento de Ingeniería Agroindustrial, Universidad Nacional del
Santa, Av. Universitaria s/n Urb. Bella Mar, Distrito de Nuevo Chimbote,
Departamento de Ancash, Perú
[3]*Current address:* Universidad Tecnológica Equinoccial,
Campus Quito, Avenida Occidental y Mariana de Jesús,
Facultad de Ciencias de la Ingeniería
*E-mail: gabriela.vernaza@gmail.com**

Brazil is the second largest soybean producer in the world, representing 25% of the total world production. Soy has become an important agricultural commodity in the Brazilian economy, because of the application of soy ingredients in a variety of market products and due to their functional properties, as well as biological and health benefits. Consumption of soybean has been linked to cholesterol reduction and prevention of cardiovascular and gastrointestinal diseases, cancer, diabetes and obesity. The health benefits of soy are attributed to the presence of bioactive compounds such as isoflavones, saponins, lunasin and others. It is possible to improve the nutritional value of soybeans when the seeds are submitted to certain processes such as germination. Germination can cause significant changes in the biochemical characteristics of seeds and during this process storage proteins can be degraded by proteases, improving their digestibility; antinutritional factors can be reduced and oligosaccharides hydrolyzed.

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Introduction

The soybeans belong to the family *Fabaceae* (leguminous plant) originally from China. Its cultivation began by the year 2800 BC and the seed was considered sacred as rice, wheat, barley and millet. It was disseminated in Western countries, mainly because it is an important source of oil for human and animal consumption. It is presented as an important source of protein, although it is still underused in the human diet and industrial products. There are a lot of products that can be achieved from soybeans such as, flour, oil, bran, soluble extract and textured protein (*1*).

The soybeans came to Brazil from the United States in 1882. The first register of soybean cultivation in Brazil dates from 1914 in Santa Rosa, RS. It gained economic importance only during the 1940's, meriting the first national statistical record in the Agricultural Yearbook of RS in 1941. However, driven by the policy of subsidies to the wheat and aiming at self-sufficiency, the soybean crop was established as economically important to Brazil in the decade of 1960. In that period, its production was multiplied by five (from 206.000 tons in 1960 to 1.056 million tons in 1969) (*2*).

Brazil ranks second in soybean production (68.5 million tons), with approximately 25% of all soybeans grown in the world, which reached about 68.5 million tons in 2010, behind only the U.S., with 90.6 million tons. Other major producers are Argentina, China, India, Paraguay and Canada (*3*).

The crops in Brazil are both conventional and transgenic soybean, although the organic soybean crops can be found, too. The organic soybean is grown free of chemicals such as herbicides, fungicides and insecticides and is also a good investment for small producers, since the organic soybean in Brazil is sold, on average, $ 250 a ton, while the conventional soybean around $ 175 a ton (*2*).

Although Brazil produces much of the conventional soybean, there is also the transgenic soybean crop. The best known and the most cultivated in the country is that plant which has obtained with biotechnology techniques a gene from another organism able of making it tolerant to a type of herbicide, the glyphosate. Glyphosate is a product commonly used by farmers to control weeds and clean areas before planting. Molecules of glyphosate bind to a vital protein of the plant, inhibiting its development and leading to death. This gene was extracted from a soil bacterium known as *Agrobacterium* and patented as *CP4-EPSPS* by a private company. It is structurally very similar to the genes that make up the genome of a plant. When inserted into the soybean genome, has made the plant resistant to the herbicide application, thus facilitating its cultivation and improving marketability (*2*, *4*).

Bioactive Compounds of Soybean with Health Benefits

Consumption of soybean has been linked to prevention of cardiovascular, gastrointestinal, cholesterol reduction, cancer, diabetes and obesity (*5*–*8*).

The bioactive compounds can be divided into proteins such as lunasin, Bowman Birkman inhibitor (BBI) and lectin, and the non-protein as isoflavones and saponins.

Lunasin

The lunasin is a single peptide originally isolated from soybeans and subsequently barley, which can prevent some types of cancer (*9*). Soybean lunasin contains 43 amino acid residues and nine aspartic acid residues at its extremity (*10*). It was discovered by Alfredo Galvez in 1996 as a result of his investigation into the nutritional profile of soy protein in the laboratory of Dr. De Lúmen, at UC Berkeley. This peptide is found in small amounts in soybeans and soy-based foods. It blocks cell division by binding to specific chromosomal proteins called "deacetylated histones" (*11*). Its effectiveness against oncogenous chemical compounds was first demonstrated in cell cultures of rat skin (*12*). The soybean lunasin presented great potential as a new anticancer agent (*9*).

Bowman-Birk Inhibitor (BBI)

The Bowman-Birk Inhibitor (BBI) has 71 amino acid residues, molecular weight of 7.975 kDa, and seven disulfide bonds in the molecule. It inhibits the activity of trypsin and chymotrypsin stoichiometrically at 1 molar ratio, in an independent and simultaneous manner (*13*). BBI is stable within the pH range encountered in most foods, withstands the temperature of boiling water for 10 min, resists to acidic pH and proteolytic enzymes in the gastrointestinal tract, is bioavailable and non-allergenic. Protease inhibitors, in general, are not considered bioactive ingredients for fortifying food due to its low specificity. The most bioactive compounds act in prevention of diseases by inhibiting the enzyme that catalyzes the pathological process. For this reason, BBI fits the definition of a functional food.

In particular, the role of protease inhibitors of a food source such as BBI is recognized by biomedical researchers as potential chemopreventive agents (*14–16*), specifically in cases of breast cancer.

Lectins

Lectins or hemagglutinins can be detected and characterized by their capacity to agglutinate erythrocytes (*17*). These effects are produced by the ability of lectins to bind to specific types of sugars on the cell surface (*18*). In addition to these properties, lectins can promote mitogenic stimulation of lymphocytes and cancer cell agglutination (*17*).

Lectins are accumulated in the seed proteins storage vacuoles and cotyledons and are degraded during germination of the seeds (*19*). The lectins have several anti-nutritional properties, but also anticancer properties (*20*, *21*) In case of studies in humans they are used as therapeutic agents, binding to cancer cell membranes or their receptors preferentially, causing cytotoxicity and leading to cancer cell agglutination and / or aggregation (*20*). Some dietary lectins can cause a chemopreventive effect in breast cancer of humans by inhibiting cell growth and proliferation in vitro (*22*).

Saponins

Saponins are triterpenoids naturally found in different plant foods. They are secondary metabolites of plants containing a steroid or triterpenoid aglycone with a number of carbohydrate molecules linked by ethers and esters connections to one or more glycosylation sites. Seeds of soybeans (Glycine max L. Merrill) contain between 0.6% to 6.5% (db) of triterpenoid saponins depending on the variety, year of cultivation, place of culture, and degree of maturity. The saponins have antifungal, antiviral, spermicidal, expectorant, diuretic, anti-inflammatory (*23*) and hypocholesterolemic activity (*24*).

Isoflavones

The soybeans and its derivatives have great potential in the functional food market due to the presence of bioactive compounds such as isoflavones, which have been widely studied for their beneficial biological effects on human health, such as estrogenic (*25*) antiestrogenic (especially on symptoms of the climacteric syndrome and osteoporosis) (*26*), antifungal (*27*) hypercholesterolemic (*28*) and anticarcinogenic properties, which have been proven in Asian populations, because of their high intake of soy (*29*). These biological properties are predominant when the isoflavones are present in the aglycone form (without glucose) instead of β-glycosides (combined with glucose) (*30*, *31*).

Isoflavones are a subclass of flavonoids, which belongs to the group called phytochemicals, compounds not included as nutrients but have been getting attention from researchers due to its estrogenic properties and prevention of cancer and other chronic diseases. Flavonoids include all the phenolic compounds from a plant and its basic structure consists of two benzene rings linked by a heterocyclic pyran ring. Soybeans are the only nature source which contains large amounts of isoflavones, above 3 mg / g on a dry basis. The original isoflavones present in soybeans are genistein, daidzein and their β-glycoside conjugates. The glycitin and glycitein also appear in smaller amounts (*32*).

The concentration of isoflavones in soybeans is genetically controlled and influenced by environmental conditions and the most important factor is the temperature during grain development (*33*, *34*). The presence and concentration of isoflavones in soybean products depend on the processing conditions and on the temperature in which the material is treated (*35*).

Bioactive Compounds in Brazilian Soybeans

There are few studies that report the content of bioactive compounds of different Brazilian soybean cultivars. Paucar-Menacho et al. (*36*) compare the bioactive compounds of a low-protein (BRS 133) soybean in comparison to a high-protein (BRS 258) soybean cultivar and concluded that the high-protein soybean contained 17% lower carbohydrates and a lower chemical score (63) in relation to the low-protein soybean, which had a higher chemical score (76), associated with the higher methionine content (1.2%). Cultivar BRS 258 had more calcium (15.5%), phosphorus (30.1%), iron (18.7%), copper (9.0%) and zinc

(11.5%), and a higher concentrations of lunasin, BBI and lectin (20.3%, 19.0% and 27.1%, respectively) than the low-protein cultivar. BRS 133 had 75.4% higher concentration of total isoflavones (5.1% of total aglycones) and 31.0% total saponins, as compared to BRS 258. It was concluded that the low-protein soybean cultivar contained higher isoflavones and saponins, but lower levels of minerals and bioactive peptides, such as lunasin.

Mandarino et al. (*37*) studied the isoflavone content of different Brazilian soybean cultivars developed by Embrapa Soja Brazil and concluded that BRS 133 showed the highest content of isoflavones, while BRS 258 and BR 36 showed the lowest content. Avila et al. (*38*) evaluated the isoflavone content of different Brazilian soybean cultivars (BR 36, EMBRAPA 48, BRS 133, BRS 184, BRS 213, BRS 214) and concluded that the BRS133 and EMBRAPA 48 cultivars showed the highest isoflavone content (168.20 and 185.13 mg/100 g, respectively), while the BR 36 cultivar showed the lowest (87.30 mg/100 g).

Alezandro et al. (*39*) evaluated the isoflavone content (daidzein and genistein) of the transgenic Brazilian cultivar BRS 243 RR and concluded that the daidzein (44.1 $\mu g.g^{-1}$) and genistein (37.4 $\mu g.g^{-1}$) contents were similar to those of conventional soybean determined in other studies. This means that the glyphosate, the herbicide widely used in soybeans did not modify the composition of the phenolic compounds such as isoflavones.

Avila et al. (*40*) evaluated the isoflavone content of different conventional and transgenic Brazilian soybean cultivars before and after 180 days of storage. Twenty one soybean cultivars: CD 202, CD 206, CD 208, CD 213RR, CD 214RR, CD 215, CD 216, CD 217, CD 218, CD 221, BRS 184, BRS 185, BRS 214, BRS 244RR, BRS 245RR, BRS 246RR, BRS 255, BRS 257, BRS 258, BRS 261 and BRS 262 were assayed. The authors concluded that all cultivars showed a higher total isoflavone content before storage. Nevertheless, exceptions were observed for cultivars CD 206, CD 214 RR, BRS 214, BRS 246 RR, BRS 257, BRS 261 and BRS 262, which presented greater isoflavone content after storage, while the lowest isoflavone contents were predominantly identified for the conventional cultivars CD 206, CD 221 and BRS 255 before storage and for BRS 255, BRS 258 and CD 221 after storage. There was no relationship between the transgeny for glyphosate resistance and the increase in isoflavone content.

Soybean Germination

The soybeans have a high nutritional value, which is determined by its protein composition. However, the seed has antinutritional factors in its structure, which may interfere with availability of certain nutrients, resulting in growth inhibition, hypoglycemia or damage to tissues such as pancreas or liver. Among these constituents, stand out the phytic acid and trypsin inhibitors (*41*).

To improve the nutritional value of soybeans and use as human food is necessary to remove or inactivate these undesirable constituents. The creation of cultivars by genetic manipulation, which contain little or no amount of these undesirable constituents is an alternative, but require long-term studies on chemical and biochemical nature of these compounds and the consequences in agronomic crop yield, tolerance to the ground, need for light and water and pest resistance (*42*). Other ways of reducing undesirable components are the processes such as grinding, hydration, cooking, fermenting, solvent extraction and germination (*43*, *44*).

The germination process has been proposed as an alternative to improve the nutritional quality of soybeans (*44*). Reductions in levels of phytic acid depending on germination time and cultivar are reported by several authors (*43*, *45*).

The effects of germination in soybean seeds on chemical composition, biochemical constituents and antinutritional factors can vary greatly with the germination conditions (temperature, light, humidity and time), cultivars or varieties of seeds and analytical methods (*46*). The development of food products from soybean germination may be another way to further increase the versatility and utility of this grain.

Effects of the Germination Process in Brazilian Soybean Bioactive Compounds

There are few studies that report the effects of the germination process in Brazilian soybean bioactive compounds (*31*, *47–49*).

Ribeiro et al. (*31*) studied the β-glucosidase activity and isoflavone content in the radicles and cotyledons of soybeans of Brazilian cultivar BRS 213 for 72 hours at 25 ° C, with samples collected and analyzed every 6 hours. They observed that the soybean germination affects the activity of β-glucosidases and the total isoflavones and their isomeric forms. The authors also demonstrated that, β-glucosidase activity is increased in the radicle and cotyledon during germination period, while the total isoflavone content was increased in the cotyledons and decreased in the radicles. Thus, changes in isoflavone content will depend on the stage of germination of seeds and its metabolism and physiology.

Paucar-Menacho et al (*47*, *48*) germinated two different Brazilian soybean cultivars BRS 133 and BRS 258 and evaluated the germination time (12-72h) and the temperature (18-32 °C) using the Response Surface Methodology with a 2^2 central composite rotational design and concluded that germination time and temperature had a significant influence on the composition and concentration of bioactive compounds in the germinated soybean flour from the Brazilian soybean cultivars. Germination of soybean cultivar BRS 133 resulted in a significant increase in lunasin (73.62%), isoflavone aglycones (daidzein and genistein) (238.36%), and total saponins (31.89%), and a significant decrease in lectin concentration (55.07%) and in lipoxygenase activity (69.92%). In the same way, germination of soybean cultivar BRS 258 resulted in an increase in the soluble protein (31.9%), isoflavone aglycones (daidzein and genistein) (153.93%) and total saponins (215.86%), while it was observed a decrease in BBI (27.0%), lectin (72.6%) and lipoxygenase activity (49.4%).

Vernaza et al. (*49*) studied the effect of germination (0h, 18h and 72h) in combination with alcalase hydrolysis (0h, 1h, 2h and 3h) of Brazilian cultivar BRS 133 flours on the production of bioactive peptides as modulators of oxidative stress and markers of inflammation. The electrophoretic profile showed a weak protein breakdown during germination while a strong breakdown of the proteins was observed after the first hour of hydrolysis with alcalase. Soybean flours presented a notable antioxidant capacity ranged from 607.85 ± 32.07 to 740.55 ± 26.10 mmol TE/g (Trolox Equivalents per gram) and it increased with germination time. All flours showed a significant inhibition on inflammatory markers such as nitric oxide (20.5 - 69.3%), iNOS (22.8 -93.6%), PGE2 (64.0 - 88.3%), COX-2 (36.2 - 76.7%), TNF-α (93.9 – 99.5%), and IKK-α (24.5 - 76.6%) in LPS-induced RAW 264.7 macrophages. However, flours with 18 h of germination were more potent in inhibiting pro-inflammatory responses when compared to 72 h.

Soybean-Derived Products Found in the Brazilian Market

Several factors related to soybean varieties have promoted the growth of soy-based products available on the market, such as increased production, advancing technologies and functional claim regarding the proteins and the presence of bioactive compounds.

Soy Flour, Concentrates, Isolates, and Textured Soy Protein

The four major types of soy protein products are: flour, concentrates, isolates, and textured soy protein. Soy flour is made through milling defatted soy meal or dehulled whole beans, with the removal of hulls and/or fat, so its protein content is similar to the starting material, about 55% on a dry-matter basis (db), it has the lowest cost among soy protein products, as it involves the least processing. It retains most nutrients from the original beans and is an excellent source of protein, isoflavones, other nutrients and phytochemicals. Soy protein concentrate is made by aqueous alcohol extraction or acid leaching of defatted soy flakes. This process removes soluble carbohydrates, and the resulting product has about 70% (db) protein. Soy protein isolate is produced by alkaline extraction followed by precipitation at an acid pH. It is the most refined soy protein product after removal of both soluble and insoluble carbohydrates. Therefore, it has a protein content of 90% (db). Textured soy proteins are made mainly by thermoplastic extrusion usually manufactured in a single-screw extruder, in which high-quality defatted soy flour with a nitrogen solubility index above 50% is moistened with water or steam (or both) to about 33% before extrusion at approximately 155°C. Cross-linking reactions between aligned denatured protein molecules form a fibrous meat-like structure that can be hydrated and used as a meat extender in a variety of foods. Control of the physical properties of this type of product involves unique die designs to impart layering, density control and the desired appearance. The textured soy proteins come in many sizes, shapes, colors, and flavors, depending on the ingredients added and the processing parameters (*50*, *51*).

These products are not consumed directly as food, but are versatile ingredients to be incorporated into many different food systems, such as: bakery, dairy, meat, breakfast cereals, beverages, infant formulas, etc. They increase protein content and also add technological functional properties, which include solubility, water absorption and binding, viscosity control, gelation, cohesion, adhesion, elasticity, emulsification, fat absorption or repulsion, flavor binding, foaming, whipping, and color control (*51*).

Table 1. Former soy protein product specifications, according to CNNPA Number 14

Product	*Moisture (max. %)*	*Protein* (N x 6.25) (min. %)*	*Fat* (max. %)*	*Fiber* (max. %)*	*Ash* (max. %)*	*PDI (min. %)*
Defatted soy flour	9.0	50.0	2.0	4.0	6.5	45
Textured soy protein	8.0	50.0	2.0	4.0	6.5	-
Soy protein concentrate	8.0	68.0	1.0	5.0	5.0	5.0
Soy protein isolate	6.0	88.0	0.5	1.0	6.0	6.0
Soy extract (liquid)	93.0	3.0	1.0 (min. %)	-	0.6	-
Soy extract (powder)	3.0 (min. %)	41.5	13.5 (min. %)	-	7.0	-

* dry basis (d.b.)

Brazilian Legislation

In Brazil, Resolution RDC Number 268 of the National Health Surveillance Agency (ANVISA) (*52*), of 22 September 2005, sets identity and minimum quality characteristics for protein products of vegetable origin.

Protein products of vegetable origin are defined as foodstuffs obtained from protein parts of vegetable species, which can be presented in granule, powder, liquid, or other forms, except those that are not conventional for foods. They can be added of other ingredients, if this does not alter product characterization.

The products must be designated as “Protein” or “Extract” or “Flour”, according to their minimum protein content, followed by the common name(s) of the vegetable species of origin.

The products must present in their composition the following protein contents:

- Textured soy protein: protein (d.b.) (N x 6.25) - minimum 50.0 % (g/100 g);
- Soy protein concentrate: protein (d.b.) (N x 6.25) - minimum 68.0 % (g/100g);
- Soy protein isolate: protein (d.b.) (N x 6.25) - minimum 88.0 % (g/100 g);
- Soy extract: (i) powder: protein (d.b.) (N x 6.25) - minimum 40.0 % (g/100 g); (b) liquid: protein (N x 6.25) - minimum 3.0 % (g/100 g);
- Defatted soy flour: protein (d.b.) (N x 6.25) - minimum 45.0% (g/100 g);
- Hydrolyzed vegetable protein: protein (d.b.) (N x 6.25) - minimum 25.0 % (g/100 g);

Former CNNPA Number 14, of 28 June 1978 (*53*), was much more detailed, setting identity and quality standards for: (i) defatted soy flour; (ii) textured soy protein; (iii) soy protein concentrate; (iv) soy protein isolate and (v) soy extract. Maximum moisture, fat, fiber and ash contents, as well as minimum PDI (Protein Dispersibility Index) were included in product specifications, as can be seen in Table 1. Color, taste, odor and aspect were defined, as well as microbiological standards.

Alternative Products Found in the Brazilian Market

Beside the four major types of soy products (flour, concentrates, isolates, and textured soy protein), in the Brazilian market it can be found alternative soybean-based product, as shown in Figure 1 and as described below.

Soybean Oil

Soybeans contain about 20% oil, which is composed mainly of triglycerides. Soybean oil has a large amount of polyunsaturated fatty acids (PUFA), such as linoleic acid (55%) and α-linolenic acid (8%). Linoleic and α-linolenic acids are considered essential fatty acids and part of the family of omega 6 and omega 3 PUFA, respectively. However, the presence of lipoxygenase in crude soybean oil promotes more rapid rancidity. The minor components found in crude soybean oil are the phospholipids also called lecithins, as well as tocopherols and phytosterols (*54*). For use in foods, crude soybean oil is refined.

Lecithin

Soybean oil is composed of 1-3% phospholipids, known as lecithins, which comprise about 35% phosphatidyl choline, 25% phosphatidyl ethanolamine, 15% phosphatidyl inositol, 5-10% phosphatidic acid. In general, lecithins are removed from the oil during the degumming process and are used as emulsifiers in the food industry (*54*).

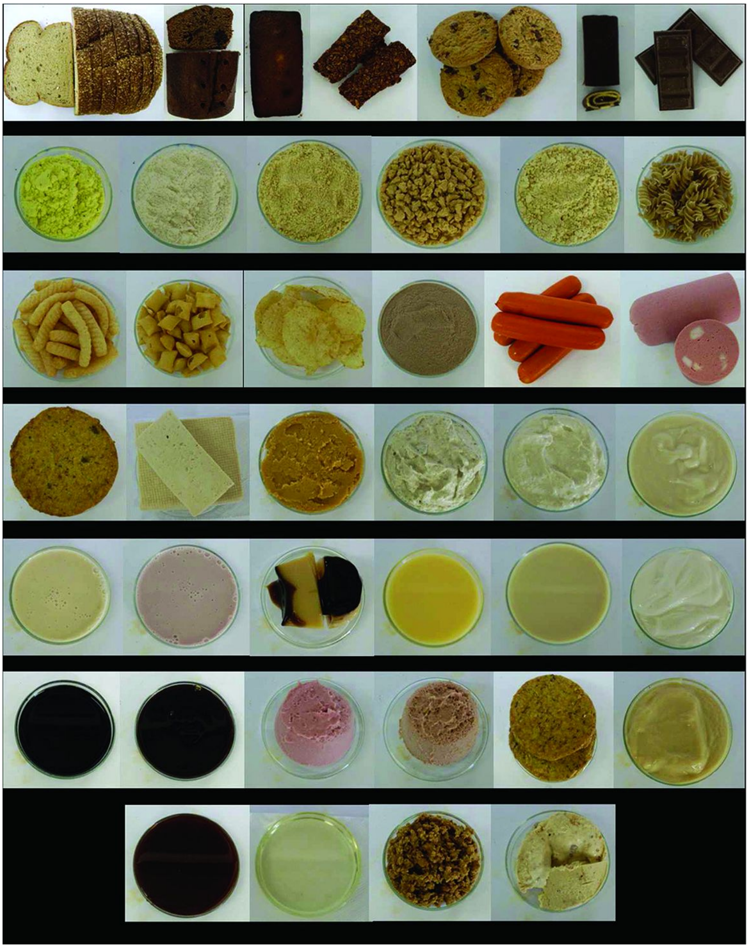

Figure 1. 1 – Bread; 2 and 3 – Cake; 4 – Cereal bars; 5 – Cookies; 6 – Chocolate roll; 7 – Soy chocolate; 8 – Soup powder; 9 – Pre mixer for tapioca cake; 10 – Defatted soy flour; 11 – Coarse texturized soy protein; 12 – Powder soy extract; 13 – Fusilli pasta; 14 – Shrimp flavored snacks; 15 – Barbecue flavored snacks; 16 – Onion and shoyu flavored snacks; 17 – Chocolate flavored milky meal; 18 – Sausage; 19 – Bologna mortadela; 20 – Green olives soy hamburger; 21 – Tofu; 22 – Miso; 23 – Eggplant flavored soy paste; 24 – Green olives flavored soy paste; 25 – Banana and apple flavored soy yogurt; 26 – Plum flavored soy fermented beverage; 27 – Strawberry flavored soy fermented beverage; 28 – Caramel flavored flan; 29 – Orange flavored water soluble soybean extract; 30 – Water soluble soybean extract; 31 – Soybean cream (like milk cream); 32 – Sauce (Shoyu); 33 - Soy condensed (like sweetened condensed milk); 34 – Ham flavored pâté; 35 – Ham and green olives flavored pâté; 36 – Organic soy hamburger; 37 – Fruit and ginger soy salad dressing; 38 – Soy lecithin; 39 - Soy oil; 40 – Canned vegetal meat; 41 – Caramel flavored ice cream.

Water Soluble Soybean Extract - Tou-Chiang

The water soluble soybean extract is comprised of components leached from the water extraction by emulsion and aqueous suspension during the process of maceration (with sodium bicarbonate), grinding, filtration and heat treatment, at a water:soybean ratio of approximately 10:1 (w/w). It is considered a product with strong aroma, bitter and astringent taste due to the action of lipoxygenases, saponins and aglycones. To minimize these negative effects, the water soluble soybean extract (WSE) is flavored, homogenized and fortified to soften the strong flavor. In most cases, the WSE is flavored and sucrose and salt (NaCl) are added to improve the aroma and taste and the main flavors available are vanilla, peach, strawberry, apple, mango, pineapple, grape and chocolate. In addition, the product is submitted to UHT processing and aseptic packaging to extend shelf life. Besides the liquid extract, WSE may also be presented in dried form. According to ANVISA (National Health Surveillance Agency) Brazilian (Resolution) RDC 268 (September 22, 2005) (*52*), soybean liquid and dried extracts must have a minimum protein content of 3 and 40%, respectively.

Tofu

Tofu is the best known derivative of soybeans and is also called soy cheese. It is obtained from the water soluble soybean extract by the addition of salts or acids to promote the precipitation of proteins at the isoelectric point. Tofu is a protein gel with smooth, soft and elastic texture. The salt most widely used for the protein precipitation is calcium chloride and glucono-δ-lactone is the acid commonly employed in the process (*55*, *56*). When $CaCl_2$ is used, the final product presents a considerable amount of calcium in its composition.

Yuba

Yuba comes from the drying of the film formed on the surface of the soybean extract when heated. It is composed primarily of protein (55%), lipid (28%), carbohydrates (12%) and ash (2%). Heating temperature of the soybean extract around 95 ° C in alkaline pH increases the yield of the film formed and the protein concentration. The polymerization of soy protein is a function of disulfide bonds and hydrophobic interactions and hydrogen bonds to a lesser extent. Dried yuba is slightly brittle, has a relatively long shelf life, and is used as a cover film or as an ingredient in a variety of dishes, for example in soups (*57*).

Okara

The residue obtained from the production of WSE and tofu is called okara and presents approximately 25-28% protein, 9-11% lipids, 4-5% carbohydrates and 52-58% of total dietary fiber (*58*). Guermani et al. (*59*) reported the fraction of total dietary fiber is composed of 12.1% hemicellulose, 5.6% cellulose, 1.7% lignin and 0.16% phytic acid. The okara is widely used in the baking industry.

Meat Product Applications

Processed meat products usually contain added soy products and soy protein isolate has been used as an ingredient in the meat industry. Fermented or emulsified isolates can improve the quality of processed meats due to their good water retention capacity and emulsifying properties. Soy protein isolate contains nitrite and nitrate in its composition, and therefore requires special processing in products as roast beef, chicken and turkey breast, beef patties, chicken patties and nuggets, pizza topping, meatballs, and meatloaf. They can improve the slicing properties, reduce purge, enhance firmness, and reduce shrinkage of injected meat products. The soy protein isolate powder may be added to the formulation during processing or may be applied as a gel, similar to ground meat.

Isolate soy protein can be used in chopped meat such as burgers, sausages, fish and structured foods. ISP shows excellent performance to emulsify fat, promotes product structural integrity, improves texture and retains water in the final product.

For products with low amounts of lean meat and ISP contents above 3%, it is necessary to use ISP with high PDI values due to its high gelling properties.

Salami and pepperoni are dry fermented meats. The soy protein isolate to replace the lean meat can be used in the production of these meat products, thereby reducing the cost or replacing part of the fat for the production of reduced-fat products. Soy protein isolates used in this application require very high gelling properties (*60*).

Meat Extender and Analogue Products

Meat extenders are obtained by thermoplastic extrusion at low moisture contents (20-35%) and meat analogues are obtained by thermoplastic extrusion at high moisture contents (50-70%). The raw materials commonly used to produce meat extenders are defatted soy flour and soy protein concentrate (SPC), whereas for the production of meat analogues, soy protein concentrate (SPC) and soy protein isolate (SPI) are used (*61*).

There are four types of meat analogues: fine emulsions (franks, hotdogs, and bologna types), coarse ground-type products (patties, links, and nuggets), crumble, strip, or chunk types (ground beef, chicken, or beef-type strips), and emulsions with particulates (chicken, bacon, luncheon meat and ham type products). A major challenge in developing products which replace meat is related to properties of texture and flavor to resemble meat.

Onchom (Ontjom)

Onchom is very similar to tempeh produced from the fermentation of okara by *Neurospora sitophila* (30°C at 80 to 85% humidity for 72 h) which produces orange-red spores. A carpet of brilliant orange-red mold should cover the surface of traditional onchom, when it is ready for consumption. Onchom deep fat fried in coconut oil has a delicate mushroom-like flavor, with overtones of a sweet-nutty flavor and aroma.

Kinako or Doufen

Known as kinako in Japan or doufen in China, the product comes from the grinding of whole soybeans after roasting and grinding processes. The kinako differs from the full-fat soy flour due to the presence of seed coat and the pronounced nut flavor.

Miso

There are various types of miso in Japan, and it is classified into three types: rice miso, barley miso, and soybean miso depending on the kind of koji. Koji is obtained from steamed grains and mold spores is placed on a large porous plate or cloth or in a wooden container through which temperature- and moisture-controlled air is passed to provide appropriate conditions for mold growth and the production of the enzymes (*62*). Soybean miso is produced by the hydrolysis of soy proteins into peptides and amino acids by the enzymes produced during soybean fermentation of koji. The use of rice to obtain miso is optional; in this case the rice starch is hydrolyzed by α-amylase and maltase. The lipids are also digested by lipases present (*63*). In general, the production of miso occurs in the presence of NaCl (12-13%).

Soy Sauce – Shoyu

Among the various types of shoyu produced, the main is oikuchi-shoyu, characterized by its deep brown color, pH around 4.7, and contains 17% NaCl, 1.5-1.6% total nitrogen, 3% reducing sugar and 2-3% alcohol. The preparation process consists of three steps: preparation of koji, fermentation in brine and refining. In the first step, a mixture of steamed soybean and roasted wheat is inoculated from 3 to 4 days by *A. oryzae*. After that, the brine is added to facilitate halotolerant yeast fermentation by *Z. rouxii*, and then by *C. versatilis* and lactic acid bacteria. The fermentation takes place for 6-8 months. The palatability of shoyu is correlated with the high content of amino acids (7.5%) and salt (17%) and the glutamic acid is the more pronounced amino acid present (*64*). Another product widely consumed in Brazil is light shoyu, due to its reduced salt content.

Tempeh

Tempeh is a food produced with different strains of *Rhizopus* spp. by solid-state fermentation. The enzymatic activity of this mold causes significant decomposition of the polymeric compounds, as well as considerable changes in flavonoids. Thus, tempeh provides excellent digestibility and confers protection against diarrhea and chronic degenerative diseases. The production of tempeh involves peeling of soybean, followed by maceration in water and briefly cooking with mold. Then, it is incubated for approximately 2 days at 30 °C. The final product can be marketed fresh or in a dried form (*65*).

Natto

Natto is a product obtained by fermentation of the soybeans under aerobic conditions. After selection and washing process, the soybeans are soaking until the mass increases by 2.2 times. Maceration process takes place in 10-14h at 20-22° C. Then the steeped soy beans are drained and heated under pressure of 0.8 to 1.0 kg/cm2 for 30-40 min. Soon after, the beans are inoculated with *Bacillus subtilis* or *Bacillus natto* at 45°C until the final process of fermentation when the grains are brightly colored and covered with grayish white film of *B. natto* (*66*).

Sufu

Sufu is obtained by fermentation of fresh tofu by certain fungi (*Mucor hiemalis* or *Actinomucor elegans*) resulting in a final product which presents texture similar to cheese. The product consists of tofu cubes covered with white or yellowish-white fungous mycelia; it has firm texture, salty taste, and characteristic flavor. The preparation methods vary with the regions where it is produced; however, it involves three basic steps: preparing bean curd, molding (first fermentation) and second fermentation. The type of fungus used for the fermentation process has great influence on the quality of the final product, once it must produce white or yellowish-white mycelia to ensure an attractive appearance to sufu. Several types of sufu can be made through uses of different processing methods and addition of different color and flavor agents. For example, the choice of processing conditions may result in mold-fermented sufu, naturally fermented sufu, bacterial fermented sufu or enzymatical ripened sufu, which can provide a red, white or gray sufu (*66*).

Douchi

Douchi is a fermented product from soybeans and black soybean is preferably used. The soybeans are washed and soaked for 3-4 h to absorb 1.8 to 2.0 times their weight in water. After this step, they are drained, mashed and steamed under pressure at 113-117 °C for 20-30 min. If soybeans have been defatted, the process of steaming can be carried out at 131 °C for 5 min. If the cooking water takes place, the temperature must be 70-90 °C. After cooling, the soybeans are mixed with roasted wheat flour (1-2%) and inoculated with a washed starter koji at 38-40 °C from 6-24 h in the presence of 17-20% NaCl. During the fermentation process, proteins are hydrolyzed to peptides and free amino acids and carbohydrates converted to lower molecular size compounds (*67*).

Fermented Beverages

The lactic fermentation of water soluble soybean extract to obtain a final product similar to yogurt has been growing in recent years to replace animal products, especially for more extreme vegetarians. However, this product provides high mineral deficiencies, particularly calcium, despite the good acceptance by consumers. The great advance in fermented soy beverages is due to the facility of probiotic food production, since the oligosaccharides, amino acids and peptides present in water soluble soybean extract provide an excellent vehicle for bifidobacteria microflora, protecting these microorganisms from bile salts and favoring intestinal colonization (*68*).

Other Products

Besides the various products mentioned above, many other foods can be produced from soybeans or by soybean added. In the bakery industry, it is very common to find products that have been fortified with soy-based products such as breads, biscuits, fresh pasta, dried and instant cakes, confectionery and cereal bars. One of the more traditional bakery products is the multi-grain soybean bread.

Extruded and expanded products are processed by addition of soy protein to increase the levels of protein, especially when using rice flour as basic ingredient for the preparation of crispy rice snacks.

Frozen desserts can be improved by adding soy protein, but the ISP must be highly soluble in water, free of flavor and have excellent emulsifying properties and high viscosity characteristics.

Due to their high emulsifying and stabilizing effects, soy proteins are also widely used in soups and sauces for improving viscosity, mouthfeel and texture.

Soybean paste is a product similar to the Brazilian cheese *requeijão* and is available in various flavors such as eggplant, carrots, black olives, leeks, parsley and chives, garlic and onion.

A traditional snack of soybeans is widely consumed. For its process, the soybeans after boiling are roasted or fried, seasoned and can be eaten as snacks in substitution for salted peanuts, nuts or walnuts.

Dairy products such as yogurts can be processed by addition of soy protein to increase the protein content and provide textural characteristics. The soybean proteins improve gel stability and increase water retention, thus preventing syneresis.

The soybean protein-based edible films as coverings for fruits have been widely studied; however, these films provide lower moisture barrier due to the protein hydrophobicity and the common plasticizers used to increase flexibility of the film matrix.

Besides the large variety of soybean food products, soybeans are widely used in Brazil in animal feed for ruminants and non-ruminants due to the high protein biological value and the low production costs of soybeans. Another sector which has gained significant market share is the biofuel sector, using soybean oil for making biodiesel as a renewable source of energy.

Conclusion

The consumption of soybean-based products is increasing in Brazil because of their health benefits and also because of their technological properties. It is expected that new soybean-based products appear on the market in the next years and it is important to teach or educate the population about the benefits of soybean consumption.

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Chapter 17

Hibiscus sabdariffa L.: Phytochemical Composition and Nutraceutical Properties

Luis Mojica, Li Rui, and Elvira Gonzalez de Mejia[*]

Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, 1201 W. Gregory Dr., Urbana, IL 61801
[*]E-mail: edemejia@illinois.edu

Hibiscus sabdariffa (HS) is a plant cultivated around the world for food uses, its medicinal properties and health benefits. Infusions of the calices of this plant are consumed for their unique taste and pleasant color either hot as tea or cold as a refreshing beverage. There are reports in the scientific literature about the effects of *H. sabdariffa* on decreasing the risk of hypertension, diabetes mellitus, inflammation, cancer and obesity, among other health conditions, due to the presence of biologically active compounds. The purpose of this chapter is to review the scientific evidence in support of the potential health effects of HS, using studies performed *in vitro*, and in preclinical and clinical models. The results suggest that biologically active compounds in HS extracts are responsible for the potential prevention of the various disease conditions associated with the consumption of HS extracts.

Introduction

Hibiscus sabdariffa (HS) is a tropical plant that is used around the world. It has been cultivated for food uses and medical purposes in Asia, Africa and several regions of South America. HS calices are used as food ingredient in salads, beverages and jams and as colorant. The plant is a rich source of antioxidants. The seeds of HS have the highest antioxidant capacity than any other part of the plant (*1*). Several researchers have worked with extracts of HS to test their effects on health and their antioxidant capacity. Numerous studies have reported that the active compounds found in this plant may help to prevent diseases such as cancer,

In Hispanic Foods: Chemistry and Bioactive Compounds; Tunick, M., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

inhibit inflammatory process, promote diuretic ativity, modulate the formation of adipose cells, decrease serum cholesterol, control diabetes mellitus, lower blood pressure, reduce kidney problems, inhibit angiotensin converting enzyme (ACE), reduce serum uric acid, and in addition, anticonceptive and antidiarrheal effects, among others. Therefore, the objective of this chapter is to review the scientific studies on the beneficial effects and safety of *Hibiscus sadbariffa,* using different experimental models.

Origin and Botanical Characteristics of Hibiscus

It is a short-day annual shrub originated from West Africa. *Hibiscus sabdariffa* is also known as "Roselle", "African mallow" and "Sour tea" in English speaking countries, as "L'oiselle" in France, "Jamaica" or "Flor de jamaica" in Spanish speaking countries, "Karkade" in Sudan and Arabia, "Rorrel" in the Caribbean and "Byssap" in Senegal. It has been cultivated for food uses and medical purposes in Asia, Africa and several places in South America. Figure 1 shows the phenotypic differences in leaf and calyx shape for twelve genotypes of *Hibiscus spp* cultivated in Mexico.

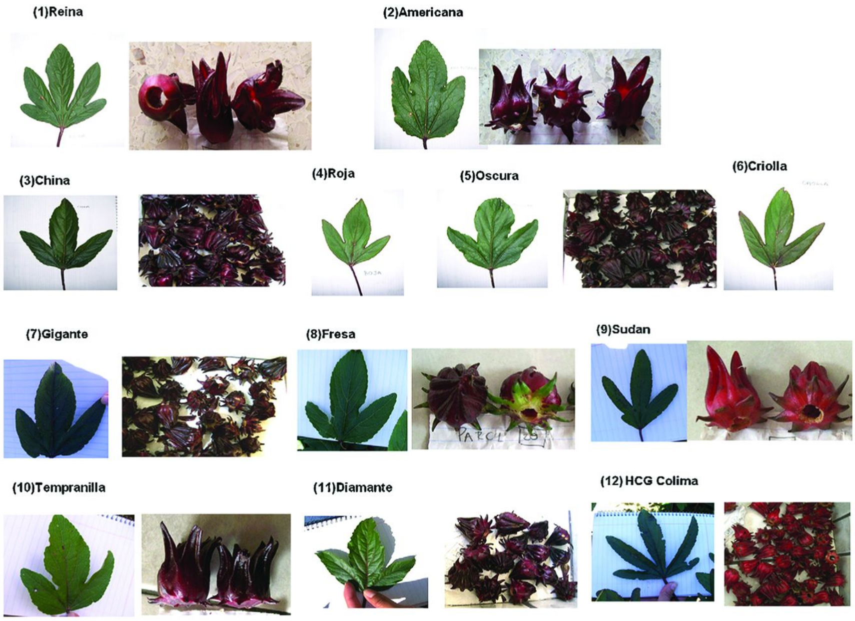


Figure 1. Phenotypic differences in leaf and calyx shape for 12 genotypes of Hibiscus spp. (Reproduced with permission from reference (51). Copyright 2011 Elsevier.)

Uses and Applications of Hibiscus

HS flower is used as food ingredient in salads, beverages and jams. The traditional way to prepare HS for consumption is to chop the flower or calices and add it to salads or chop and boil it to make jams and sauces. The flower/calices can also be dried and boiled to make infusions to be consumed either as hot teas or cold beverages (*2*). In Mexico, refreshing beverages made from the flower of HS is very popular, especially during the summer. Sepals are the parts most used for herbal remedies. It has been used in various regions as a laxative and as a medical food for the treatment of diseases. The pharmacological activities include treating inflammation, bacterial infection, diabetes, high blood pressure, hypercholesterolemia, kidney diseases, and some types of cancer.

Phytochemical Composition

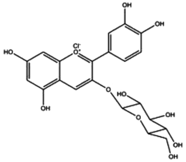
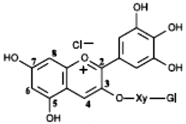
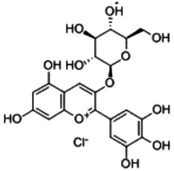
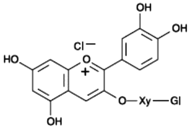
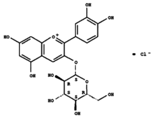
The therapeutic value of HS is the result of active ingredients in the plant. The flower contains high concentration of flavonoids and polyphenolic compounds, such as anthocyanins. These phytochemicals possess antioxidant, antipyretic, and analgesic activities (*2*). The flower is a rich source of water-soluble polysaccharides, which contributes to the dietary fiber content of the Mexican diet as those compounds are disperse in the beverage made from HS. It is also a good source of organic acids, including citric, malic, tartaric, oxalic and ascorbic acids. The plant also contains minerals, such as calcium and iron, and vitamins, such as niacin, riboflavin, and vitamin C. Oil from the seeds has an anti-infection effect. A list of significant phytochemicals is provided in Table 1.

Hibiscus sabdariffa is commonly extracted with cold or boiling water (*3*). The highest antioxidant capacity of extracts from calyx and fruits of the plant was shown in 30% ethanol extracts compared with 60% and 95% ethanol extracts (*4*). Segura-Careetero (*5*) used solid phase extraction-capillary electrophoresis-mass spectrometry method (CE) to separate and qualitatively determined the content of anthocyanins in Hibiscus.

Antioxidant Capacity

One of the most recognized health benefits of HS is its antioxidant potential. Consuming natural antioxidants can protect the body against free radicals and reactive oxygen species, which increase the oxidative stress and induce several kinds of diseases. Various antioxidant phytochemicals are found in the calyx and flower petals of the plant, including high concentrations of phenolic compounds and anthocyanins, and the presence of other compounds, such as ascorbic acid, steroid glycosides and protocatechuic acid. The phenolic ring and hydroxyl substituents of phenolic phytochemicals are similar to the structure of vitamin E, and are able to neutralize hydroxyl and its related free radicals (*6*). Yang (*4*) showed that 30% ethanol extract retained the strongest antioxidant activity.

Table 1. Phytochemical composition of Hibiscus parts

Organic compound	Molecular Structure	Origin	References
Anthocyanin:			
Cyanidin-3-glucoside		Calyx	11,47.48
Delphinidin-3-glucoxyloside (hibiscin)		Calyx	11,47,48
Delphinidin-3-monoglucoside		Calyx	47,48
Cyanidin-3-sambubioside		Calyx	11,47, 48
Cyanidin-3-monoglucoside		Calyx	48

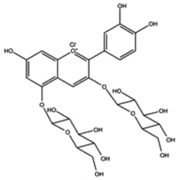
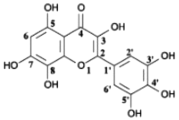
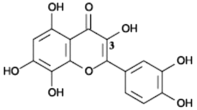
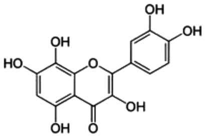
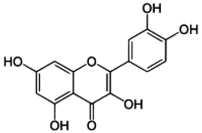
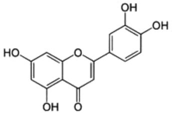
Organic compound	Molecular Structure	Origin	References
Cyanidin-3,5-diglucoside		Calyx	48
Hibiscetin		Calyx	11
Gossypitrin		Calyx	48
Gossypetin		Calyx	48
Quercetin		Calyx	11,47,48,49
Luteolin		Calyx	49

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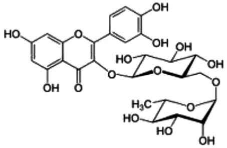
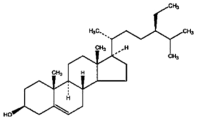
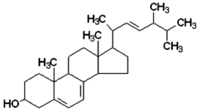
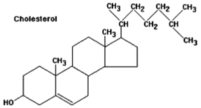
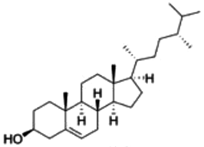
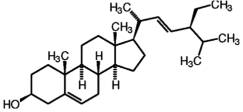
Table 1. (Continued). Phytochemical composition of Hibiscus parts

Organic compound	Molecular Structure	Origin	References
Rutin		Calyx	11
Sterols:			
Beta-stosterol		Seed oil	47, 48
Ergosterol		Seed oil	47, 48
Cholesterol		Seed oil	47,48
Campesterol		Seed oil	47, 48
Stigmasterol		Seed oil	47, 48

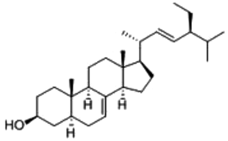
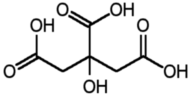
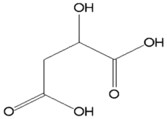
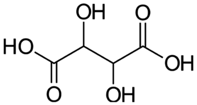
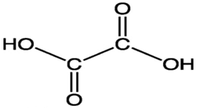
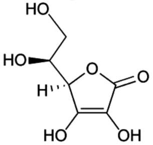
Organic compound	Molecular Structure	Origin	References
Alpha-spinasterol		Seed oil	47, 48
Organic acids:			
Citric acid		Calyx	47, 48
Malic acid		Calyx	47, 48
Tartaric acid		Calyx	47, 48
Oxalic acid		Calyx	47
Ascobic acid		Calyx	48

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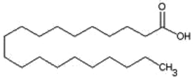
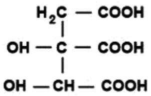
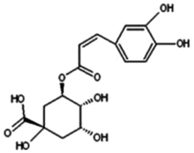
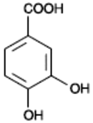
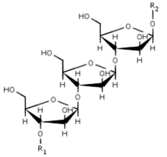
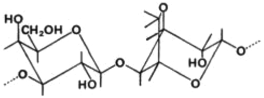
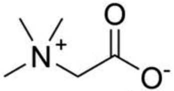
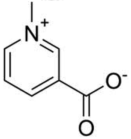
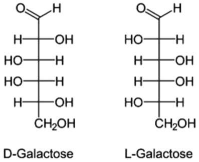
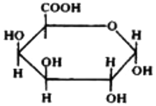
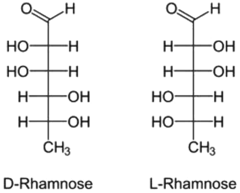
Table 1. (Continued). Phytochemical composition of Hibiscus parts

Organic compound	Molecular Structure	Origin	References
Arachidic acid		Calyx	48
Hibiscus (hydroxycitric) acid	 $H_2C-COOH$ $OH-C-COOH$ $OH-CH-COOH$	Calyx	47
Chlorogenic acid		Calyx	47
Protocatechuric acid (PCA)		Calyx	50
Water-soluble polysaccharides:			
Arabinan		Calyx	47
Arabinogalactan		Calyx	47

Organic compound	Molecular Structure	Origin	References
Alkaloid:			
Glycinebetaine		Calyx	47
Trigonelline		Calyx	47
Mucilage hydrolysis products:			
Galactose	 D-Galactose L-Galactose	Petal	48
Glacturonic acid		Petal	48
Rhamnose	 D-Rhamnose L-Rhamnose	Petal	48

The levels of antioxidant activity differ among *Hibiscus sabdariffa* parts and the calyx of the HS flowers has been recognized as a source of antioxidant compounds, such as vitamin C, anthocyanins, β-carotene, lycopene, polyphenols and other water soluble antioxidants (*7*). Calyx extracts show strong antioxidant activity both *in vitro* and *in vivo*. HS seed extracts were found to have the highest antioxidant activity and strongest radical-scavenging activity (*7*). The seeds contain considerable amount of phenolics, and the presence of phytosterols and tocopherals also contributed to the antioxidant activity of the plant. The antioxidant activity was evaluated using β-carotene bleaching method, DPPH radical-scavenging examination, and measurement of effectiveness in the reduction of lipid oxidation in cooked beef patties. Overall, water extracts of HS plant parts showed an order of strength of antioxidant activity as: calyces > seeds > leaves > stems; whereas methanol extracts showed an order as: seeds > calyces > leaves > stems. Consumption of HS aqueous extract caused significant increased in the systemic antioxidant potential and reduced the oxidative stress in humans (*8*). In this study, eight healthy individuals were treated with an aqueous HS extract or a reference treatment with water. The results showed significant increase in plasma antioxidant power (FRAP), increased ascorbic acid (the biomarker for oxidative stress), and hippuric acid (metabolite and potential biomarker for total polyphenol intake), while malondialdehyde excretion was reduced. The reducing power of anthocyanin from HS extract was examined by Ajiboye (*9*). The results showed that anthocyanins had approximately 2-fold higher reducing power than a synthetic antioxidant, butylated hydroanisole. Moreover, the nonenzymic antioxidants, such as vitamin C and vitamin E, were also significantly preserved in the extract.

Diuretic Activity

Diuresis is an important process for excreting catabolites, maintaining the hydroelectrolyte equilibrium and eliminating toxic substances (*10*). The diuretic effect of HS has been studied (*11*), as a side-effect in renal disease investigations (*12–14*) and also when studying herb–drug interactions of this plant with diuretic drugs (Table 2). Diuretics work by promoting the elimination of urine and urinary sodium from the body and this helps to reduce the volume of blood circulating through the cardiovascular system (*15*). HS as diuretic has been pharmacologically studied in preclinical experiments (*11–13*, *16*). Alarcon-Alonso (*11*) found an increase in urinary volume with doses of 1500, 2000 and 2500 mg/kg of HS extract in rats and a clear and consistent natriuretic effect (increased urinary excretion of sodium). Woottisin (*12*) and Laikangban and Devi (*13*) studied antilithic effect and urolithiasis respectively in a rat model. In the case of Woottisin group they found a significant increase in urine volume at week 4 vs baseline. The rats fed with 3% glycolic acid and 3.5 mg tablet of HS extract showed significant increase of oxalate in urine. Laikangban and Devi (*13*) reported that the curative and prophylactic treatment with aqueous extract of HS caused diuresis and hastened the process of dissolving the preformed stones and prevention of new-stone formation in the urinary system with doses of 250, 500 and 750 mg/kg. On the other hand, Ndu (*16*) and Prasongwatana (*14*) reported no volume increase in clinical trials and preclinical trials, respectively. Ndu (*16*) reported that only co-admistration of HS (40 mg/kg) and hidrochlorothiazide (HCT) (10 mg/kg) caused a significant increase in the volume of urine in a rat model. Prasongwatana (*14*) reported that the diuretic effect of HS herbal tea was not observed at the dose of 3 g/day in volunteers.

Antihypertensive Activity

High blood pressure is a global health problem with significant morbidity and mortality (*17*). Approximately 900 million people throughout the world suffer from hypertension (HT); of these 420 million reside in developing countries. It has been calculated that 1.5% (17 million) of those patients die each year due to causes directly related with HT. The main objective of medical treatment for HT is to avoid disease progression, prevent cardiovascular complications, maintain a good quality of life and reduce the mortality associated with this disease, while blood pressure (BP) should be < 140/90 mm Hg. Different factors play an important role in the pathogenesis of HT. Among the best known factors are water and sodium retention in the vascular compartment and increased activity of the plasmatic ACE, which results in an increase in angiotensin II production and activity (*18*). The pharmacological agents used to treat hypertension are ACE inhibitors, and diuretics (*17*). The use of extracts of HS, as part of the diet, showed its efficiency in the reduction of BP in clinical trials with hypertensive patients (*19–23*). A significant decrease in blood pressure over the controls was reported (Table 3). Similarly, studies of the antihypertensive effect of HS in rats showed experimental evidence of the beneficial effect of HS extracts. Mojiminiyi (*23*) reported a BP reduction in models of salt sensitive HT rats and HT rats due to chronic nitric oxide synthase (NOS) inhibition. Ajai (*24*) found that HS extract induced a vasodilator effect in isolated aortas from spontaneously hypertensive rats via endothelium-dependent and –independent vasodilator pathways. This explains the BP lowering effect of HS in vivo, and provided further evidence to the traditional use of this plant as an antihypertensive agent.

Antiadipogenic Activity

Obesity and overweight are chronic conditions characterized by excess body fat that is quantified by the elevation in body weight. Recently, the prevalence of overweight and obesity has increased worldwide at an alarming rate and represents an important public health problem. It is accepted that overweight and obesity result from a disequilibrium between energy intake and expenditure and these conditions have a large impact on several metabolic and chronic ailments including heart disease, cancer, arthritis, obstructive sleep apnea, hypertension, hyperlipidemia, and diabetes type II associated with insulin resistance (*25*). Adipose tissue is recognized as the major energy depot in higher eukaryotes. Its prominent purposes are storing triglyceride in periods of energy excess and its mobilization during energy deprivation. Weight loss reduces lipid levels, blood pressure, and the incidence of type 2 diabetes mellitus. New drug therapies that can be used to reduce the prevalence of obesity that produce weight loss with minimal adverse effects have been used (*26*). The effect of HS on obesity has been widely investigated using in vitro, preclinical and clinical models. Most studies showed a significant decrease in markers of lipid metabolism (Table 4). Kim (*26*), Hernandez-Lopez (*27*) and Kao (*28*) studied the effect of HS extract and polyphenols from HS on 3T3-L1 preadipocyte cells and macrophage J774A.1 cells in in vitro models. Kim (*26*) found that HS extract can inhibit adipogenesis in different ways at the concentration of 2 mg/ml. On the other hand, Kao (*28*) found that the treatment of J774A.1 cells with HS extract (0.05–0.2 mg/ml) largely prevented lipid accumulation in these cells. Hernandez-Lopez (*27*) worked with HS aqueous extract and a purified extract (PEHS) which inhibited lipid accumulation at concentration of ≥ 500 µg/ml for HS and PEHS at concentration < 10 µg/ml. Both extracts showed a dose-dependent response in the reduction of accumulation of triglycerides. Alarcon-Aguilar (*25*) reported that HS extract suppressed body weight gain in obese/MSG mice and reduced glycemia at a concentration of 120 g/kg/day. Carbajal-Zabarral (*29*) found that body weight gain in rats was significantly less at concentrations of 10, and 15 g/100 g/day of HS extract than control. Fernandez-Arrollo (*30*) and Yang (*31*) reported that HS extracts showed hypolipidemic activity with a reduction of serum triglycerides and hepatic fat accumulation in mice and hamsters under hypercaloric diets. Some authors have tested anti-adipogenic capacity of HS extract in clinical trials with volunteers (*32–34*). However, the different levels and methods of extraction of polyphenols from the HS caused variations in results. Mohagheghi (*34*) studied the effect of HS on serum lipids in a group of patients that had to take two cups of HS hot tea per day. Results did not show significant changes in cholesterol and triglycerides. Gurrola-Diaz (*32*) used a freeze-dried HS extract in volunteers with metabolic syndrome (MeSy) and found a hypolipidemic and hypotensive effect with a preventive diet and HS powder extract (1.42 mg / kg) (*32*). The effect on human serum cholesterol was significantly decreased with a dose of 2 capsules per day (1000 mg/daily) (*33*).

Inflammatory Inhibition Effect

Chronic inflammation is associated with several human pathologies and causes the up-regulation of several pro-inflammatory proteins in affected tissues. Among the numerous proinflammatory enzymes, inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) produce nitric oxide (NO) and prostaglandin E2 (PGE2) in lipopolysaccharide (LPS)-activated macrophages and in other stimulated cells. These enzymes are involved in initiating obesity, cardiovascular disease, neurodegenerative disease, diabetes, and cancer. Cancer may arise from chronic irritation and inflammation or, conversely, an oncogenic change can induce an inflammatory microenvironment that promotes the development of tumors (*35*). Acute inflammation is part of the defense response, but chronic inflammation has been found to mediate a wide variety of diseases. Chronic inflammation accompanied by oxidative stress is linked to various steps involved in tumorigenesis, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis (*36*). Beltran-Debon (*37*), Ali (*38*) and Kao (*36*) have studied the anti-inflammatory effect of HS extract *in* *vivo* and *in vitro* models (Table 5). They found in all cases that HS has a significant effect in the prevention of inflammation. Beltran-Debon (*37*) reported that HS extract effectively protected peripheral blood mononuclear cells (PBMCs) from the cellular death induced by H_2O_2 modulating the production of inflammatory cytokines. On the other hand, the extract caused 18% and 27.5% inhibition of ear edema formation at the doses of 250 and 500 mg/kg, respectively (*38*). HS prevented inflammation, besides its anti-oxidative profile, was capable of impairing COX-2 induction by down-regulating JNK and p38 MAPK proteins (*36*).

Antidiabetic Effect

Diabetes mellitus (DM) is a highly prevalent disease worldwide with a consistent increase in mortality, and accompanying vascular complications including coronary heart disease, nephropathy and neurodegeneration. Diabetes-associated metabolic syndrome is manifested as hyperglycemia, high triacylglycerol (TG), and low high density lipoprotein (HDL) dyslipidemia. For type II diabetes, which is characterized as insulin-resistant and often associated with overproduction of free fatty acids (FFA), obesity is suggested to be a critical morbidity factor in the pathogenesis (*39*). DM has been defined by the World Health Organization as a fasting plasma glucose concentration greater than 7.8 mmol/l (140 mg/dl) or greater than 11.1 mmol/l (200 mg/dl) 2 h after a carbohydrate meal or 2 h after an oral ingestion of the equivalent of 75 g glucose, even if the fasting concentration was normal (*40*). DM is a consequence of chronic metabolic aberrations including hyperlipidemia. High glucose facilitates glycolysis and adenosine triphosphate generation would cause huge reactive oxygen species (ROS) production. Excessive oxidative stress could damage proteins, lipids, and DNA and eliminate anti-oxidative enzymes (*41*). Some authors have studied the effect of HS extract on different indicators of disease in preclinical models using diabetic rats (*39–43*) (Table 6). Wang (*41*) reported that HS extract had the capacity of decreasing TG and LDL-C significantly in streptozotocin (STZ) diabetic rats. Sini (*39*), found that the extract caused significant decrease in serum total cholesterol and triacylglycerol from diabetic levels in alloxan induced diabetic rats. HS extract also demonstrated its protective effect under high-glucose conditions. It has been shown that HS inhibited LDL oxidation and reduced foam cell formation and migration of VSMC (vascular smooth muscle cells) (*42*). It had a clear effect on anti-insulin resistance properties, which demonstrated effects on hypoglycemia and reversed the hyperglycemia in preclinical models (*39*, *43*).

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Table 2. Diuretic effect of Hibiscus in different models

Model used	***Objective***	***Extraction method***	***Dose***	***Effect on urinary volume***	***Ref***
Male Albino rats	Diuretic effect	Water extract 55°C (2 h), lyophilized	Control, 1500, 2000 and 2500 mg/kg.	Urine excretion increased significantly from the basal volume.	(*11*)
Male Wistars rats	Antilithic effect	Water 72°C (1 h), lyophilized	Control, 3% of glycolitic acid, glycolitic acid + 3.5 mg HS extract.	Urine volume significantly increased at week 4 vs baseline.	(*12*)
Albino rats	Herb–drug Interaction on diuresis	Methanol (96 h), vacuum evaporated	Control, HCT (10 mg/kg), HCT (10 mg/kg) + distilled water, HS (40 mg/kg), HCT (10 mg/kg) + HS (40 mg/kg).	Coadmistration of HS with HCT caused a significant increase in the volume of urine, HS did no cause increase in urinary volume.	(*16*)
Male Albino rats	Urolithiasis	10 g in 100 ml of water, centrifugated (10 min), decanted and filtered, stored a 4°C	Control, urolithiatic rats 0.75% ethylene glycol and 2% ammonium chloride, urolithiatic rats cystone (750 mg/kg), urolithiatic rats aqueous extract of HS at 250, 500 and 750 mg/kg, normal rats aqueous extract of HS at 250, 500 and 750 mg/kg.	Increased diuresis.	(*13*)
Healthy males, 36 to 65 years old with no renal stones NS; and males with renal stones RS from rural communities of Khon Kaen, province Tailand	Uricosuric effect	1.5 g in 150 ml of water 98°C (10 min), the tea was provided to the volunteers twice a day.	Study performed in three steps 1: control, 2: during drinking tea, 3: after drinking tea.	Diuretic effect of this herbal tea was not observed at the dose of 3 g/day.	(*14*)

HCT, hydrochlorothiazide; HS, Hibiscus sabdariffa; NS, No renal stones; RS, Renal stones.

Table 3. Antihypertensive effect of hibiscus sabdariffa

Model used	*Objetive*	*Extraction method*	*Concentration*	*Effect on hypertension*	*Conclusion*	*Reference*
Nonsmoking men and women 30-70 y with BP problems	Determine whether consuming hibiscus tea, into the diet lowers the BP.	Brewing a bag of 1.25 g HS in 240 mL of boiled water for 6 min and artificial HS flavor concentrate as control.	720 ml/day in three servings for 6 weeks	HS tea lowered SBP by 5.5%, DBP by 4.0% , and MAP by 4.7%, whereas the placebo beverage did not affect these variables	Daily consumption of 3 servings of HS tea, into the diet, effectively lowered BP in pre- and mildly hypertensive adults.	(*19*)
Patients with hypertension 25-65 y	Compare the antihypertensive effect HS extract.	HS was macered in sterile water at 60°C for 8 h, concentrated, freeze-dried, packed in one dose.	250 mg of anthocyanis/dose in 250 ml of water during 4 weeks	HS reduced BP by 17.14 / 11.97, (11.58 /12.21%)	HS had properties of prescribed drugs for long term treatments of HT, ACE inhibitory and diuretic.	(*20*)
Patients with diabetes mellitus type II and hypertension	Short-term effects of consuming ST infusion on BP in patients with type II diabetes.	2 g of HS, in a tea pot, add 240 ml of boiling water and drink it after a steeping time of 20–30 min.	2 g of HS in 240 ml of boiling water two times/day during 1 month	SBP decreased from 134 ±11.8 to 112.7 ±5.79, PP decreased from 52±12.2 to 34.5±9.3. In DPB did not show any variation	ST infusion 2 times a day had positive effects on BP in type II diabetic patients.	(*21*)

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Table 3. (Continued). Antihypertensive effect of hibiscus sabdariffa

Model used	*Objetive*	*Extraction method*	*Concentration*	*Effect on hypertension*	*Conclusion*	*Reference*
Subjects of both sex between 30 and 80 y with hypertension	Compare the antihypertensive effect of HS extract	10 g of HS, in 0.5 l of boiling water and let stand for 10 min.	0.5 l of infusion, drink daily before breakfast for 4 weeks	The reduction of diastolic pressure rates was better in our study, reaching a 12.3% reduction.	Evidence of the innocuousness of the short- and long-term administration of HS extract.	(*22*)
Wistar male rats	Test the effects of this extract on animal models of salt sensitive hypertension and hypertension due to chronic NOS inhibition	30 g of HS brewed in 200 ml of boiled water, filtered and evaporated to dryness.	HS was administered through the femoral vein in graded doses of 1–125 mg/kg. Each dose was given after the effect of the previous dose had maximized.	BP fall in salt-loaded rats was about 1½ times the fall in control rats while the fall in rats subjected to chronic NOS inhibition was more than 2½ times the fall in control rats.	This study provides further experimental evidence that justifies the folkloric use of this plant in the treatment of hypertension.	(*23*)
Spontaneously hypertensive rats	Examine the possible mechanisms underlying the hypotensive effects of HS.	Powdered HS were subjected to soxhlet extraction with methanol, then evaporated to dryness using rotary evaporator (40°C) and freeze-dried.	Relaxant effects to HSE at different concentrations (from 10 ng/mL to 1 mg/mL) were recorded by adding cumulative concentrations of HSE to the tissue	HS extract induced a vasodilator effect in isolated aortas from spontaneously hypertensive rats via endothelium-dependent and	The present findings explains the BP lowering effect of Hibiscus sabdariffa L. in vivo, and provided further evidence to the traditional use of the plant as an	(*24*)

Model used	***Objetive***	***Extraction method***	***Concentration***	***Effect on hypertension***	***Conclusion***	***Reference***
			bath at 3 min intervals.	-independent vasodilator pathways.	antihypertensive agent.	

BP, blood pressure; Y, years; HS: hibiscus sabdariffa; SBP, sistolic blood pressure; DBP, diastolic blood pressure, MAP, mean arterial pressure; HT, hypertension; ACE, angiotensin convertin enzyme; ST, sour tea hibiscus; PP, pulse pressure; NOS,nitric oxide synthase; HSE, crude methanolic extract of the calyx of hibiscus; l, liters.

Table 4. Antiadipogenic effect of Hibiscus sabdariffa

Model used	***Objetive***	***Extraction method***	***Concentration***	***Effect on adipogenesis***	***Ref.***
Obese animal model (Ob/MSG mice)	HS effect on body weight of obese mice	Extracted twice with water (50°C), redisolved in 4 L of water, concentrated under reduced pressure.	Ob/MSG mice and healthy mice (120 g/kg/day) of Hs-extract for 60 days; Ob/MSG mice and healthy mice received 4 ml/kg of ISS for 60 days as a control.	Hibiscus sabdariffa extract suppresses body weight gain in Ob/MSG mice by 9.6% and reduces glycemia.	(*25*)
Male Sprague-Dawley rats	HS on fat absorption, excretion, and body weight	135 g of HS in 500 mL ethanol (96%) for 8 days, filtered, concentrated and stored at 4°C.	Basal diet as control and basal diet plus extract of HS at 5, 10, and 15 g /100 g for 4 weeks.	Body weight gain in SD10 and SD15 groups was significantly less than control.	(*29*)
Male LDLR mice	HS drinking fluid antihyperlipemic effects.	HS was grounded and boil in tap water, infusion was filtered. Final concentration 10 g/L.	Two diets: Chow diet and High fat diet. Two groups for diet: 1 drinking water as control, 2 drinking HS extract.	HS showed hypolipemic properties, reduction of 50% serum triglyceride under hypercaloric diet.	(*30*)
Male Syrian golden hamsters	HPE exerts the hypolipidemic effect	150 g of HS (95°C, 6000 mL) for 2 h, evaporated under vacuum at -85°C, filtered and lyophilized to obtain 75 g of HSE and stored at 4° C before use.	Purina Chow diet as control high-fat diet (HFD) with 1% or 2% HSE (w/w) for the HSE group, and HFD with HPE 0.1% or 0.2% (w/w) for the HPE group. For 10 weeks.	Hypolipidemic and hepatic fat- lowering effect of HPE suggesting that although HPE promotes LDL transport from plasma into liver, it inhibits the hepatic neo-lipid synthesis.	(*31*)
MeSy and non-MeSy volunters 30-71 years	Evaluate HSEP on the lipid profile of MeSy and healthy individuals	500 g HS in7 l of 30% ethanol (v/v) at 20 °C for 7 days, filtered evaporated at 35° C. spraydryer to obtain a poder.	Group 1 preventive treatment (diet); group 2 HSEP treatment (1.42 mg/kg); group 3 diet combined with HSEP (1.42 mg/kg) treatment.	HSEP could be a useful complement in MeSy treatment due to its hypolipidemic and hypotensive effects.	(*32*)

Model used	***Objetive***	***Extraction method***	***Concentration***	***Effect on adipogenesis***	***Ref.***
High cholesterol level volunters	Cholesterol-lowering effect of HSE	150 g HS in hot water (95°C, 6000 ml) for 2 hours, evaporated under vacuum at 85°C. Filtered and lyophilized.	G I: 1 capsule, 500 mg of HSE 3 times daily; G II: 2 capsules, 1000 mg of HSE 3 times daily; G III: 3 capsules, 1500 mg of HSE 3 times daily during 4 weeks.	HSE significantly decreased serum cholesterol in humans with 2 capsules daily.	(*33*)
Hypertensive patients	HS reduction of serum lipid concentration	15 mg of HS tea in two glasses of boiling water for about 10–15 minutes.	15 mg of HS tea in two glasses of boiling water for about 10–15 min. Drink the tea within 20 min, during one month.	Short-term administration of HS has no significant harmful changes in cholesterol, TG, BUN, serum creatinine, and Na and K levels.	(*34*)
3T3-L1 preadipocyte cells	effects of hibiscus extract on adipogenic differentiation of 3T3-L1 cells	150 g in water (95°C, 6000 ml), evaporated and lyophilized. HS powder was diluted in phosphate -buffered saline (pH 7.4) and stored at −20°C.	The cells were treated with hormone mixture MDI in the presence of various concentrations of HS extract (0, 250, 500, 1000, 2000 and 5000 μg/ml).	Hibiscus extract can inhibit adipogenesis in different ways at the concentration of 2mg/ml.	(*26*)

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Table 4. (Continued). Antiadipogenic effect of Hibiscus sabdariffa

Model used	*Objetive*	*Extraction method*	*Concentration*	*Effect on adipogenesis*	*Ref.*
3T3-L1 preadipocyte cells	Effects of polyphenols of HS on mouse adipocytes	Purified extract (PEHS) was prepared by removing fibre and polysaccharides by precipitation in 85% ethanol (v/v) from HS extract. Separed by chromatography, evaporated and lyophilized.	HS inhibited lipid accumulation at conc. of ≥ 500 μg/ml and PEHS at conc. < 10 μg/ml. Both extracts showed a dose- dependent response in the reduction in the accumulation of triglycerides.	Its observed regulation of adipogenesis, its regulation of oxidative stress signalling pathways in mature and/or hypertrophied adipocytes and its subsequent ability to alter the expression of adipokines.	(*27*)
Mouse macrophage J774A.1 cells	Anti-atherosclerotic effect of HAs	20 g HS with methanol (1% HCl for 1 day at 4°C) filtered and Conc.; then kept on an Amberlife Diaion HP-20 resin column for 24 h clean in, 0.1% HCl, eluted with methanol. Filtrate was lyophilized to obtain HAs.	J774A.1 cells are treated with various concentrations of HAs (0.01, 0.05, 0.1, 0.5 and 1 mg/ml) for 18 h. Cell viability did not significantly decrease even after treatment with 1.0 mg/ml of HAs.	Treatment of J774A.1 cells with the HAs (0.05–0.2 mg/ml) largely prevents lipid accumulation in these cells. HAs inhibits oxLDL-mediated foam cell formation and CD36 gene expression.	(*36*)

Ob/MSG, obese induced by monosodiun glutamate; HS, Hibiscus sabdariffa; l, litres; ISS, isotonic saline solution; SD10, suplement diet 10%; SD15, suplement diet 15%; LDLr, low-density lipoprotein receptor; HFE, high-fat diet; HSE, hibiscus sabdariffa extract; HPE, hibiscus sabdariffa polyphenols; LDL, low density lipoprotein; MeSy, metabolic syndrome; HSEP, Hibiscus sabdariffa extract powder; HAs, hibiscus anthocyanin; TG, trigyceride; BUN, blood urea nitrogen; Na, Sodium; K, potassium; oxLDL, oxidized low density lipoprotein, Conc, concentration; HP, Hewlett-Packard; MDI, isobuthylmethylxanthine dexamethasone insulin; HCL, hydrochloric acid; PEHS, phenolic extract of Hbiscus sabdariffa.

Table 5. Anti-inflammatory effect of hibiscus sabdariffa

Model used	***Objective***	***Extraction method***	***Concentration***	***Effect on inflamation***	***Ref.***
Healthy volunteers of both sexes (n=10)	Evaluate HSE reduction of oxidative stress and inflammation in vivo and in vitro effects in the production of selected cytokines.	650 g of calyces were heated in 5 l of boiling water for 5 min. Infusion was filtered, centrifuged and lyophilized.	(i)PBMCs were incubated with HSE and 50 mM H_2O_2 at 37 °C for 20 h; (ii) after a pre-incubation of PBMCs with HSE for 24 h and subsequent washing with D-PBS, cells were incubated with 50 mM H_2O_2 under the same conditions for an additional 20 h.	Effectively protects PBMCs from the cellular death induced by H2O2 modulating the production of inflammatory cytokines. In humans, HSE elicits a significant decrease in MCP-1 plasma concentration.	(*37*)
Swiss-albino mice	Evaluate the possible antinociceptive, anti-inflammatory and antidiarrheal activities of ethanolic extract of HS in mice	HS calyces were ground, 100 g of power was taken into 500 ml ethanol 95% for 10 days filtered and evaporated.	250 and 500 mg/kg of HS extract were administered and 0.01 ml of xylene was injected to the anterior and posterior surfaces of the right ear	It caused 18% and 27.5% inhibition of ear edema formation at the doses of 250 and 500 mg/kg respectively	(*38*)
Rat macrophage RAW 264.7 cells and Male Sprague-Dawley rats	Evaluate the anti-inflammation of polyphenol extract from Hibiscus sabdariffa L. (HPE) in vitro and in vivo	HPE extract in methanol, filtered and concentrated, Then extracted with hexane and ethyl acetate and lyophilized	The cells were treated with HPE (0–0.5 mg/ml) for 24 h. 5 groups of rats, HPE (10, 20, and 40 mg/kg) during 5 days, in day 5 LPS was (5 mg/kg) or DW was injected	HPE prevent inflammation, besides its anti-oxidative profile, is capable of impairing COX-2 induction by down-regulating JNK and p38 MAPK.	(*28*)

HSE, hibiscus sabdariffa extract; l, litres; PBMCs, peripheral blood mononuclear cells; D-PBS, dulbeccos phosphate-buffered saline; MCP-1, monocyte chemoattracant; HS, hibiscus sabdariffa; HPE, polyphenol extract from hibiscus sabdariffa; LPS, lipopolysaccharide; DW, distilled water; COX-2,cyclooxygenase-2; JNK, c-Jun N terminal kinase; p38 MAPK, mitogen-activated protein kinase antibodies.

Table 6. Antdiabetic effect of Hibiscus sabdariffa

Model used	*Objective*	*Extraction method*	*Concentration*	*Effect on diabetes*	*Ref.*
Male Sprague-Dawley rats	Assess the effect of HSE on anti-oxidation and on regulation of Akt signaling pathway in diabetic nephropathy	HS Calyx 150 g in water (95°C, 6000 ml) for 2 h evaporated, filtered and lyophilized	1-control group; 2- buffer control group,3- STZ group diabetic rats; 4- group diabetic rats fed HSE 100 mg kg−1/day; 5- group diabetic rats fed HSE 400 mg kg−1/day. During 8 weeks	HSE have the capacity of decreasing TG and LDL-C significantly in STZ diabetic rats.	(*41*)
Male Wistar strain albino rats	Evaluating hypoglycemic and hypolipidemic activities in alloxan induced diabetic rats were tested.	Powdered HS calyx 300 g in 2.5 l water at 70°C (3 h), filtered and conc. at 40°C on water bath	G1: control, G2: control rats, 0.5 mg/ml of aqueous extract solution, G3: D rats, only water, G4: D rats, water with glibeclamide 0.08 mg/kg, G5: D rats, ascorbic acid 0.2 mg/ml, G6: D rats, 0.5 mg/ml of aqueous extract a, During 5 weeks.	The extract exhibited hypoglycaemic activity in alloxan induced diabetic rat. The extract cause significant decrease in serum total cholesterol and triacylglycerol from diabetic levels.	(*40*)
Rat aortic smooth muscle cells (A7r5 cells)	Evaluate if glucose could induce VSMC proliferation and migration and the possible signals involved.	Based on Lin et al., 2007	Cells were seeded at 5 × 10^5 cells/well in a 6-well plate and obtained 50-70% confluence. An injury line was created with a single scratch at the center of a VSMC monolayer (50-70% confluence) using a sterile 1.15 mm diameter pipet tip. 24, 48, and 72 h.	HPI demonstrates protective effect under high-glucose conditions. It has been shown that the H. sabdariffa extract inhibited LDL oxidation and reduced foam cell formation and migration of VSMC	(*42*)

Model used	***Objective***	***Extraction method***	***Concentration***	***Effect on diabetes***	***Ref.***
Male Sprague-Dawley rats	Evaluate how HPE could effect changes in serum insulin, glucose, lipid profile, oxidative stress, and the characteristic biomarkers of AGE/RAGE and CTGF	HS calyx, 5 g in 50 ml methanol, 60°C water bath 30 min and filtered. Extracts pooled and evaporated. Residue solubilized in 10 ml of water and partitioned with n-hexane and ethyl acetate. Ethyl acetate soluble fraction evaporated.	G1- control, G2- normal diet with 200 mg/kg HPE; G3- FAT + HPE 100 mg/kg; G4- FAT + HPE 200 mg/kg; G5- FAT + STZ FAT + STZ + HPE (100 mg/kg); G6 FAT + STZ + HPE (200 mg/kg)	HPE demonstrated effects on hypoglycemia, hypolipidemia, and antioxidation.	(*39*)
Albino Wister rats	Investigated the hypolipidemic and antioxidant effects of ethanolic extract of Hibiscus sabdariffa L (HSE1) in rats treated with alloxan	One kg of HS was soxlet extracted with ethanol (HSE1) were concentrated in a rotary evaporator and stored in the dark at 4°C.	G1- control, G2 and 5; and 3 and 6 animals were administered daily with HSE (100 mg/kg) and HSE1 (200 mg/kg), respectively for a period of 4 weeks. G-4, 5, 6, and 7 were treated with alloxan. G7- 10 mg/kg of lovastatin, blood glucose concentrations determined at the time of sacrifice	HSE1 reversed the hyperglycemia of alloxan-treated rats.	(*43*)

HSE, aqueous extract of Hibiscus sabdariffa; Akt, protein kinase B; HS, Hibiscus sabdariffa; STZ, streptozotocin; TG, triglycerides; LDL-C, low density lipoprotein; conc, concentration; D, diabetic; VSMC, vascular smooth muscle cell; HPI, polyphenolic isolate from hibiscus sabdariffa; HPE, poliphenolic extract of Hibiscus sabdariffa; AGE; diabetes promoted plasma advanced glycation end product; RAGE, receptor of AGE; CTGF, connective tissue growth factor; HSE1, ethanolic extract of Hibiscus sabdariffa.

Table 7. Safety of Hibiscus sabdariffa

Model used	*Objective*	*Extraction method*	*Concentration*	*Toxicity*	*Ref.*
Adult Wistar albino rats	This work was carried out to evaluate the toxicity of the extract of the plant calyces on albino rats.	Powdered HS 350 g was soaked in 1000 ml of methanol-water (4:1) for 96 h, filtered, concentrated and freeze-dried	G1- Control, G2-6- received 1, 3, 5, 10 and 15 doses of 250 mg/kg each, respectively; rats were sacrificed 24 h after treatment	Prolong usage of this extract could cause liver injury but the effect was mild with small doses.	(*44*)
Male Charles Foster rats	Evaluate the effect of a 90-day oral administration of the extracts of the dried calyx of HS in an animal model.	One l of DW, water/ethanol 50:50) and 100% ethanol were used to infuse 100 g of HS powder for 4 h extracts obtained for each solvent was pooled, filtered and dried in vacuo and concentrated.	G1-6 were 300 mg/kg or 2000 mg/kg of each solvent extract daily. G7- was the control. Animals were observed for any form of morbidity and/or mortality during 90 days.	High doses of HS extracts (over 10000 human consumption) could be toxic to the hepatic system, and cause muscular dystrophy.	(*45*)
Wistar Rats	Examine both the antihypertensive and toxicological effects of HS	HS extract was concentrated in a rotary evaporator and freeze-dryed.	Administered with 1000, 3000 and 5000 mg/kg body weight dosage became less active and this effect appears to be dose dependent.	The LD_{50} is thus estimated to be greater than 5000 mg/ kg	(*46*)

HS, hibiscus sabdariffa; DW, distilled water; d, days; LD_{50} letal dose test.

Safety

Hibiscus sabdariffa has been used as a folk medicine in many cultures, without signs of toxicity. However, the toxic effect of the HS extract was evaluated in preclinical trials with rat models (Table 7). In general, in all cases the toxic effect was reached at a very high concentration (*43*). The prolonged usage of this extract could cause liver injury but the effect was mild with small doses (*44*). High doses of HS extracts could be toxic to the hepatic system, and cause muscular dystrophy (*45*). The LD_{50} was thus estimated to be greater than 5000 mg/kg and it was reasonably safe without evidence of acute toxicities (*46*).

Conclusions

The nutraceutical effect of *Hibiscus sabdariffa* has been demonstrated by different research groups in *in vitro*, preclinical and clinical models. Differences observed in its effect on the various disease conditions are mainly due to the method of extraction of the active compounds and the doses used. Based on the reviewed evidence, it is scientifically supported that the regular consumption of Hibiscus may help to control and prevent certain diseases, without health risk.

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Chapter 18

Yerba Mate (*Ilex Paraguariensis* St. Hilaire) Saponins Inhibit Human Colon Cancer Cell Proliferation

Sirima Puangpraphant,[1] Mark A. Berhow,[2] and Elvira de Mejia*,[3]

[1]Department of Food Technology, Faculty of Science, Chulalongkorn University, Payathai Road, Patumwan, Bangkok, Thailand 10330
[2]USDA, ARS, National Center for Agricultural Utilization Research, 1815 N. University St., Peoria, IL 61601
[3]Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, 228 ERML, 1201 W. Gregory Drive, Urbana, IL 61801
***E-mail: edemejia@illinois.edu**

Ilex paraguariensis St. Hilaire tree is native to South America and its dried leaves are used to prepare a traditional beverage called Yerba Mate tea. The aim of this study was to assess the anticancer properties of yerba mate saponins *in vitro* models. HT-29 (p53 mutant) and RKO (wild type p53) cells were treated with mate saponins (1- 200 μM). Mate saponins inhibited HT-29 (IC_{50} = 202 μM) and RKO (IC_{50} = 181 μM) cell proliferation. Mate saponins arrested HT-29 and RKO cells at G1 to S phase by significantly upregulating p21 and p27 proteins, and caused apoptosis through induction of Bax:Bcl-2 protein expression. Mate saponins induced apoptosis and cytotoxicity in human colorectal cancer cells independent of p53 status. Yerba mate tea saponins inhibit human colon cancer cell proliferation.

Introduction

Saponins are a class of tritepenoid or steroid compounds that are widely distributed in plants, such as in soybean (*1*, *2*), ginseng (*3*, *4*) and yerba mate (*5*, *6*). The primary saponins identified from *Ilex paraguariensis* were matesaponin 1 and 2 with ursolic acid as the triterpenoidal aglycone (Figure 1).

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ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

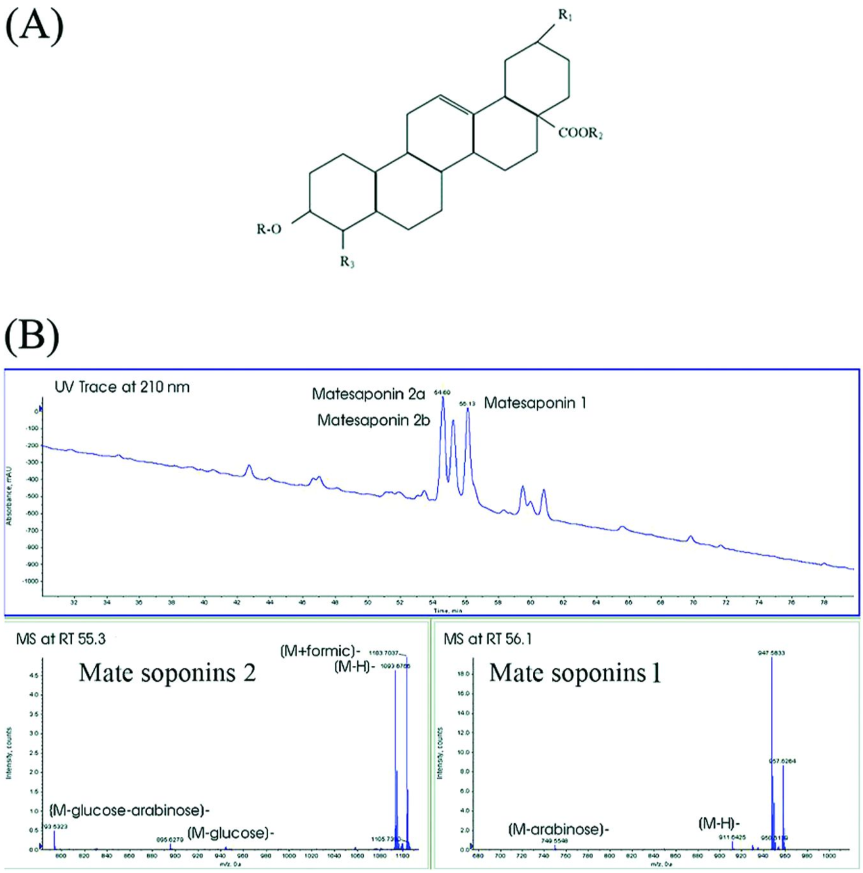

Figure 1. (A) Aglycone of saponins (ursolic or oleanolic acids) with sugars attach at R, R1, R2, or R3 (B) LC-MS of an analytical run of yerba mate saponins.

Saponins from various plant sources affect the growth of colon cancer cells. Soy saponins prevent colon cancer by affecting cell morphology, cell proliferation enzymes, and cell growth of human colon cancer cells (*7*). Tin et al. (*8*) investigated the anti-carcinogenic effect of *Astragalus* saponins in HT-29 human colon cancer cells and *in vivo* using nude mice xenografts. They found that *Astragalus* saponins induced caspase-8 extrinsic apoptotic cascade and cause cell cycle arrest by modulation of both mTOR and ERK signaling pathways, of which inhibition of NF-κB was important in the latter mechanism (*9*).

Puangpraphant et al. (*10*) demonstrated that saponins extracted from yerba mate dried leaves had anti-inflammatory activity in macrophages and inhibited HT-29 colon cancer cell proliferation by promoting apoptosis through activating caspase-3 activity. However, whether saponins from yerba mate affect the growth of cancer cells by causing apoptosis or necrosis or cell cyle arrest is still not clear.

Colorectal cancer (CRC) involves a multistep pathway of colonic mucosal genetic mutations. Over 1.2 million new cases and 608,700 deaths were attributed to CRC worldwide in 2008 making it the third and second most common cancer in males and females, respectively (*11*). One gene plays an important role in late CRC progression, the tumor suppressor gene, p53. Loss of p53 protein activity has been observed in 70-80% of colon adenocarcinomas suggesting that functional loss of p53 occurs late in tumorigenesis. Depending on the cell type and conditions, p53 regulates either cell cycle arrest or apoptosis. However, information about the mechanism of mate saponins on cell cycle arrest and mediators that regulate cell cycle, p21 and p27, and influence of p53 remains poorly understood. The aim of the present study was to determine the mechanism underlying the anti-colon cancer effects of yerba mate saponins *in vitro*. We evaluated the activity of yerba mate saponins on cell proliferation, apoptosis, and cell cycle regulations and focused on specific pathways mediated by p53 in human colon cancer cells.

Materials and Methods

Chemicals and Cells

Human colon cancer cell lines HT-29, RKO, normal colon fibroblast CCD-33Co, McCoy 5A medium, Eagle's Minimum Essential Medium and 0.25% (w/v) Trypsin- 0.53 mM EDTA and Dulbecco's Modified Eagle Medium with L-glutamine (DMEM) were purchased from American Type Culture Collection (Manassas, VA). Fetal bovine serum was purchased from Invitrogen (Grand Island, NY, U.S.A.). Bcl-2, Bax, p21, p27, p53 and actin mouse monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and antimouse IgG conjugated horseradish peroxidase secondary antibody was purchased from GE Healthcare (Buckinghamshire, U.K.). Cisplatin (> 99%) was purchase from Sigma (St. Louis, MO, U.S.A.). All other chemicals were also purchased from Sigma unless otherwise specified.

Extraction and Purification of Yerba Mate Saponins

Saponins were extracted and fractionated from yerba mate (*Ilex paraguariensis*) leaves, organically grown in Paraguay following the method as previously described (*10*). Confirmation of the identity of mate saponins in the mate extract fractions was performed by LC/ESI-MS analysis as previously described (*10*). Mate saponins were dissolved in DMSO at 10 mg/mL by weight as an initial stock and the initial stock was then diluted with culture medium to different concentrations (1- 200 μM) using the molecular weight of saponins equal to 1,000 to calculate μM equivalent concentrations.

HT-29, RKO, and CCD-33co Cell Culture and Proliferation Assay

HT-29 and RKO cells were cultured in McCoy 5A growth medium containing, 1% penicillin/streptomycin, 1% sodium pyruvate and 10% fetal bovine serum at 37 °C in 5% CO_2/95% air. CCD-33Co colon fibroblasts were cultured in Eagle's Minimum Essential Medium containing 10% FBS and 1% penicillin/streptomycin. A cell proliferation assay was performed using the MTS/PES CellTiter 96 Aqueous assay kit (Promega Corporation, Madison, WI, U.S.A.) as previously indicated (*12*). For CCD-33Co, 1 × 10^3 cells per well were seeded in a 96-well plate and allowed to grow to confluence for one week with replacement of medium every other day. For HT-29 and RKO, 5x10^4 cells per well were seeded in a 96-well plate and total volume was adjusted to 200 μL with growth medium and allowed to grow for 24 h. Both cells were then treated with different concentrations of yerba mate saponins (1- 300 μM), caffeine (≥ 99%), caffeic acid (≥ 98%), quinic acid (≥ 95%), chlorogenic acid (≥ 95%), quercetin (≥ 98%), ursolic acid (≥ 90%) (1- 300 *μ*M), and mate tea extract (1- 300 μg/mL) for 24 h. The extraction method of mate tea was previously described (*12*).

Cell Cycle Distribution of HT-29 and RKO Colon Cancer Cells

Analysis of cell cycle distribution was performed using flow cytometry. Briefly, HT-29 and RKO cells were seeded at a density of 2 × 10^5 cells per well in a 6-well plate and allowed to grow for 24 h at 37 °C in 5% CO_2/95% air. The cells were then treated with different concentrations of mate saponins ranging from 1 to 200 *μ*M for another 24 h at 37 °C in 5% CO_2/95% air. After treatment, cells were fixed overnight with 70% ethanol at 4 °C and stained with propidium iodide solution (0.1% v/v). Cell cycle distribution analysis was performed using a LSR II flow cytometer (BD Biosciences (San Jose, CA, U.S.A.) at excitation wavelength of 488 nm. Fluorescence emission was measured using a 695/40 nm band pass filter. A total of 20,000 events were collected for each sample. The analysis was performed in triplicate.

Analysis of Apoptosis of HT-29 and RKO Colon Cancer Cells

The apoptotic status of the HT-29 and RKO colon cancer cells was evaluated by determining the presence of phosphatidylserine on the cell membrane using an Annexin V-FITC apoptosis detection kit (Sigma-Aldrich, MO, U.S.A.) by flow cytometry. Briefly, 2 x10^5 cells per well were seeded in a 6-well plate and allowed to grow for 48 h at 37 °C in 5% CO_2/95% air. The cells were then treated with mate saponins (1-100 *μ*M) for 12 h at 37 °C in 5% CO_2/95% air. After treatment, cells were washed with PBS twice, trypsinized and suspended in binding buffer at a concentration of 1 x 10^6 per mL. Five hundred microliters of treated and untreated cells were transferred into a plastic test tube and stained with 5 μL Annexin V-FITC and 10 μL propidium iodide (PI) solution for 10 min. PI staining was performed concomitantly with Annexin V-FITC staining to determine whether any DNA/nuclei were present in the colon cancer cells. The cells were analyzed immediately by LSR II flow cytometer (BD Biosciences, San Jose, CA, U.S.A.). The analysis was performed in triplicate.

Western Blot Analysis of p21, p27, Bax, Bcl-2, and p53 Protein Expression

HT-29 and RKO cells were seeded at a density of 2 × 10^5 cells per well in a six-well plate for 24 h at 37 °C in 5% CO_2/95% air. After 24 h incubation, cells were treated with mate saponins (1- 200 *μ*M) for 24 h. After treatment, cells were trypsinized and suspended in lysis buffer composed of 62.5 mM Tris–HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 5% β-mercaptoethanol and protease inhibitor cocktail (Thermo Scientific, Rockford, IL). Cell suspension was then used for Western blot for p21, p27, p53, actin, Bax and Bcl-2 using antibodies (1:200).

Statistical Analysis

Data are presented as means ± SD for the indicated number of independently performed experiments. Data were analyzed using one-way ANOVA and means were considered to be significantly different at $p < 0.05$ as determined by Tukey's.

Results

Effect of Yerba Mate Saponins on Cell Proliferation of RKO Colon Cancer Cells

Figure 2 shows that mate saponins inhibited RKO colon cancer cells proliferation in a concentration-dependent manner.

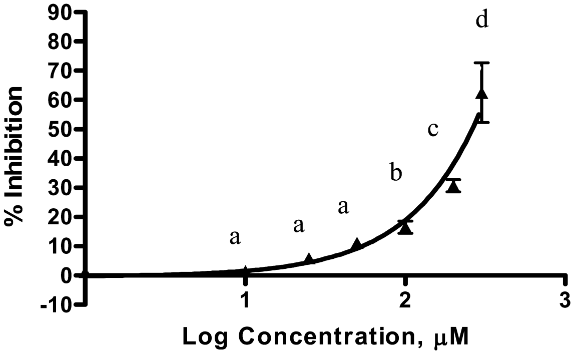

Figure 2. Dose-response curve of mate saponins on proliferation of RKO cells. Means with different letters are significantly different from each other (n = 3, p < 0.05).

The concentration of mate saponins that inhibit proliferation of HT-29 (IC_{50} = 201.8 μM) and RKO (IC_{50} = 181.0 μM) are shown in Table I. Mate saponins, mate tea extract and its constituents did not cause any cytotoxicity to CCD-33co normal colon fibroblast up to 300 μM (data not shown). The inhibition of proliferation in RKO (wild-type p53) was greater than with mate saponins-treated HT-29 (mutated p53). As shown in Table I, mate tea extract, chlorogenic acid, caffeic acid, quinic acid, and caffeine had a weak inhibition to both HT-29 and RKO cells. We also

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measured cytotoxicity of cisplatin (> 99%) which is a chemotherapeutic drug to HT-29 (IC_{50} = 80.6 μM) and RKO (IC_{50} = 68.5 μM). We found that ursolic acid was significantly the strongest anti-proliferative agent to both colon cancer cells, HT-29 (IC_{50} = 30.2 μM) and RKO (IC_{50} = 68.5 μM).

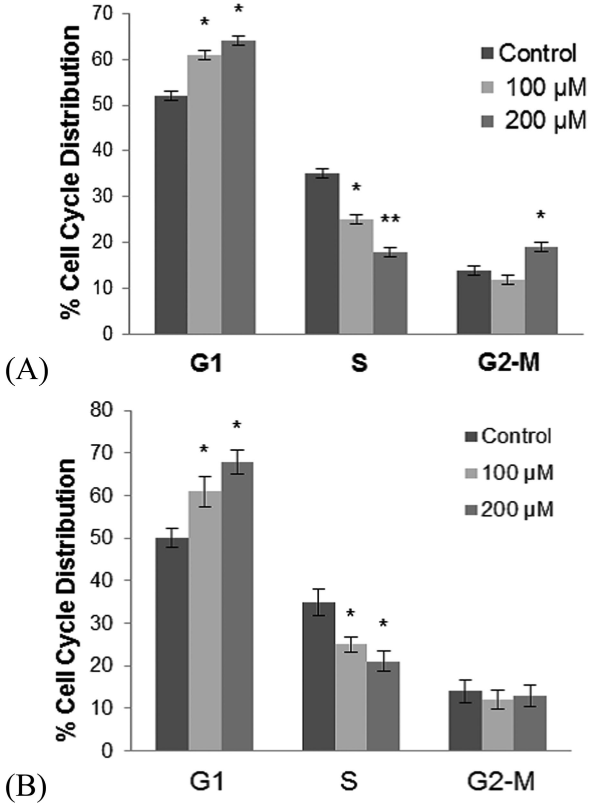

Figure 3. Cell cycle distribution (%) of cells treated with yerba mate saponins in (A) HT-29 and (B) RKO. Means with different letters are significantly different from each other (n = 3, $p < 0.05$).

Yerba Mate Saponins Induced G1 Cell Cycle Arrest of HT-29 and RKO Cells

The effects of mate saponins on cell cycle progression were studied by flow cytometry. For HT-29, p53-deficient cells, mate saponins (100 μM) significantly increased cells in G1 and decreased cells in S phase, resulting in an overall G1 to S-phase arrest (Figure 3A). Mate saponins (200 μM) also caused an increase of G2/M phase. For RKO, p53-proficient cell line, mate saponins affected cell cycle by significantly arresting at G1 to S-phase (Figure 3B).

Effect of Yerba Mate Saponins on p21 and p27 Protein Expressions in HT-29 and RKO Cells

In order to investigate the mechanism by which mate saponins inhibit the growth of HT-29 and RKO colon cancer cells, we analyzed the protein expression of p21 and p27. Mate saponins significantly increased p21 protein expression in HT-29 at 200 µM (Figure 4A) and RKO at 100 µM (Figure 4B) and upregulated p27 protein expression in HT-29 at 1 µM (Figure 4C) and RKO at 200 µM (Figure 4D), suggesting that yerba mate saponins inhibit the cell proliferation by inducing cell cycle arrest.

Table I. Yerba mate tea bioactive compounds on human colon cancer cells

Compound	*HT-29 (µM)*		*RKO (µM)*	
	$^{*}IC_{30}$	$^{*}IC_{50}$	IC_{30}	IC_{50}
Ursolic acid	16.2	30.2^{c}	13.5	29.5^{d}
Cisplatin	44.0	80.6^{b}	40.2	68.5^{c}
Quercetin	53.2	81.5^{b}	83.2	189.4^{a}
Saponins	82.3	201.8^{a}	57.5	181.0^{b}
Mate tea extract (µg/mL)	204.2	>300	198.2	>300
Caffeine	200.2	>300	200	>300
Chlorogenic acid	>300	>300	>300	>300
Caffeic acid	>300	>300	180	>300
Quinic acid	>300	>300	150	>300

* IC_{30} and IC_{50} are the concentrations (µM) that resulted in 30%, and 50% inhibition of cell proliferation (mean ± SD, n = 2). Different letters indicate significant differences, comparing differences within column, $p < 0.05$.

Yerba Mate Saponins Increased Apoptotic HT-29 and RKO Cells by Upregulating Bax:Bcl-2 Protein Expression

To determine whether the cell death of HT-29 and RKO was due to apoptosis, we treated RKO cells with mate saponins at 50 and 100 µM for 24 h. Figure 5 shows that mate saponins treatment led to significant increase on apoptotic HT-29 and RKO cells.

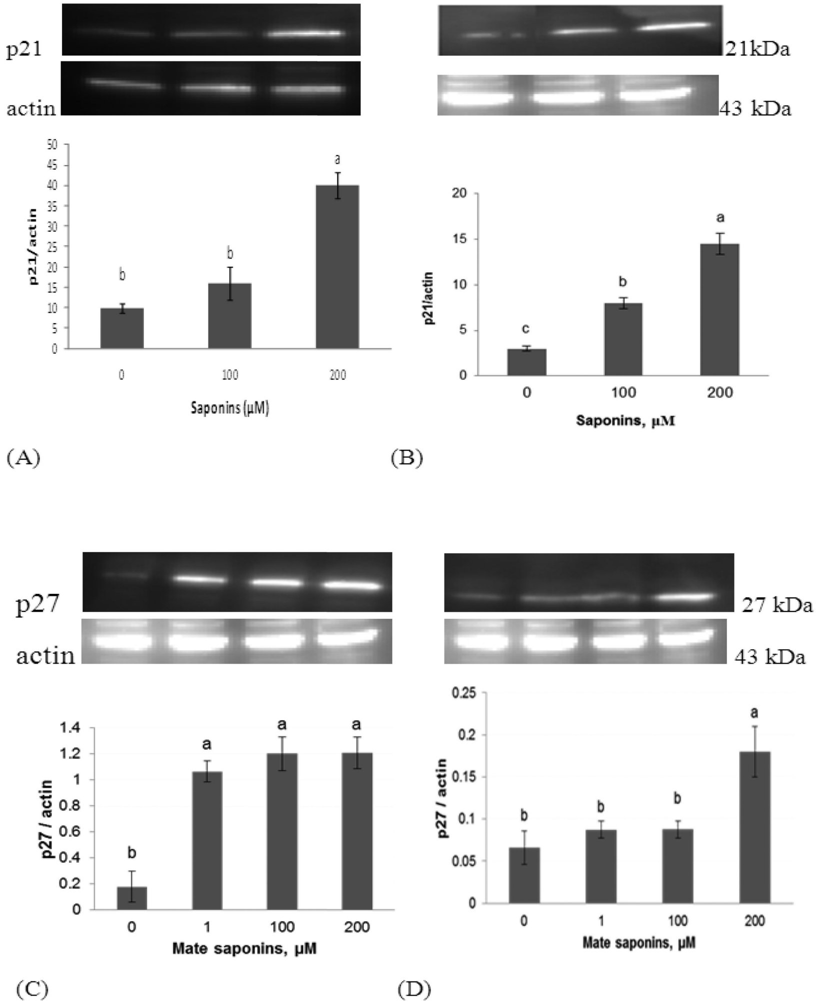

*Figure 4. Effect of different concentrations of mate saponins on protein expression of p21 in (**A**) HT-29 (**B**) RKO, and of p27 in (**C**) HT-29 and (**D**) RKO were assessed by Western blots. Actin was used as a protein loading control. The data represent the mean ± SD of a triplicate from three independent experiments. Different letters indicate significant differences, $p < 0.05$.*

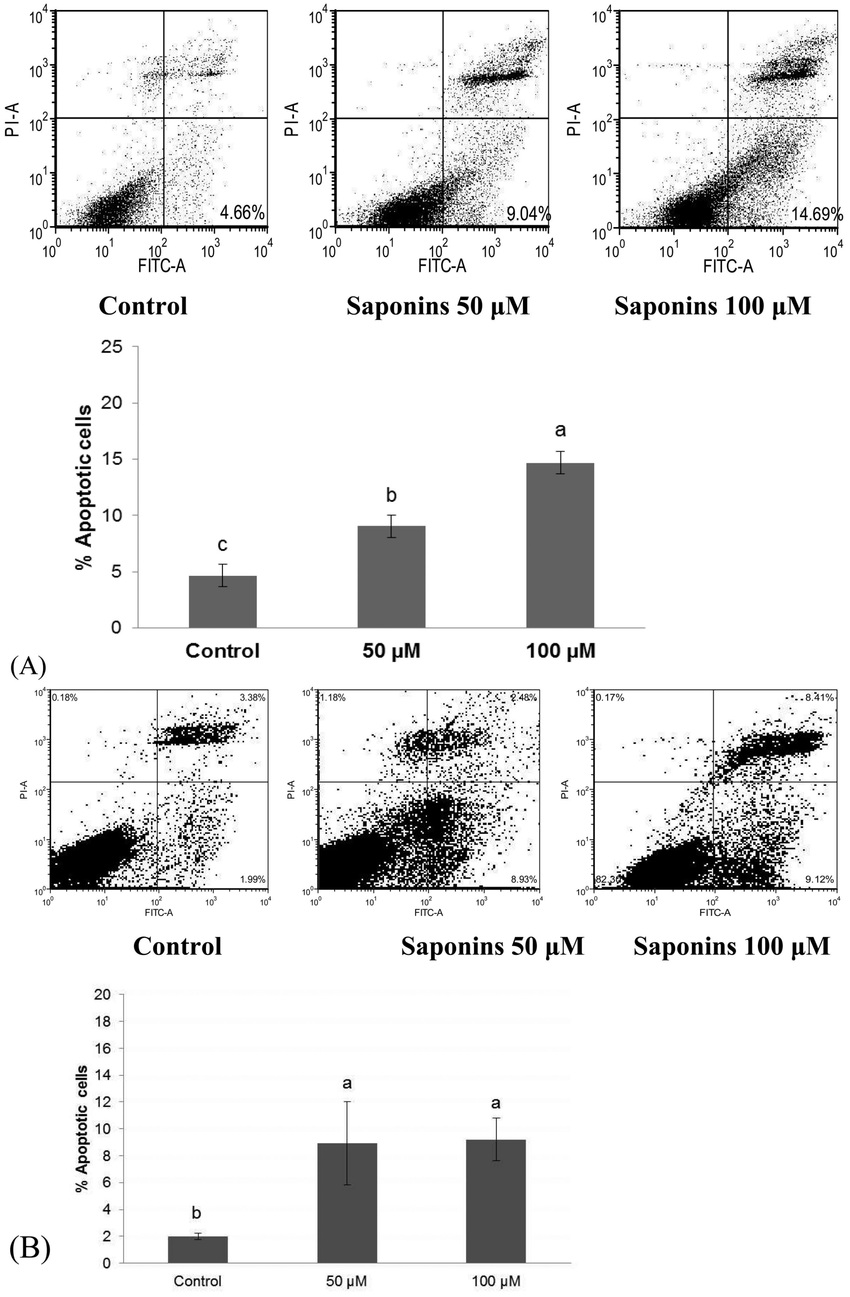

Figure 5. Effect of mate saponins on apoptotic (A) HT-29 cells; (B) RKO cells.

Mate saponins at 100 μM increased apoptotic cells from 4.7% (control) to 14.7% in HT-29, and from 2.0% (control) to 9.1% in RKO. We then analyzed induction of apoptosis by assaying the protein expression of apoptosis mediators, Bax and Bcl-2. Figure 6 shows that mate saponins significantly increased the ratio of Bax/Bcl-2 expression in RKO cells at 1 μM.

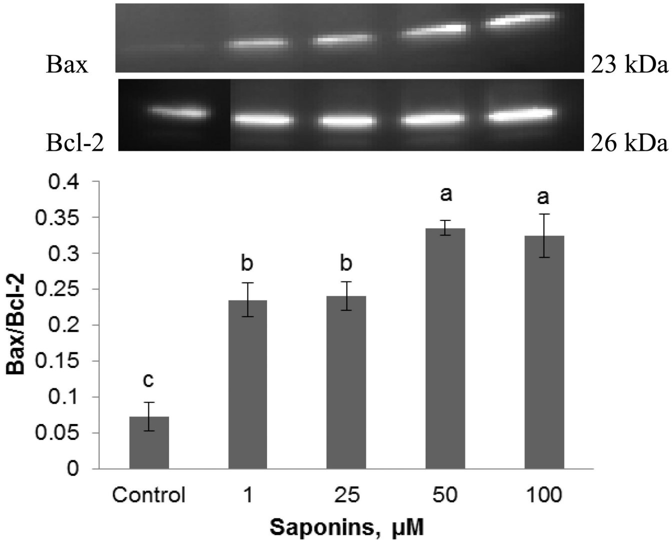

Figure 6. Effect of different concentrations of mate saponins on (A) protein expression of ratio Bax/Bcl-2 in RKO assessed by Western blots. Actin was used as a protein loading control. The data represent the mean ± SD of a triplicate from three independent experiments. Different letters indicate significant differences, $p < 0.05$.

Effect of Yerba Mate Saponins on p53 Protein Expression in HT-29 and RKO Cells

Figure 7 shows that mate saponins at 1 μM significantly induced p53 expression in HT-29 (Figure 7A) but no significant changes were observed in RKO cells (Figure 7B).

Discussion

Finding a compound that would work both in p53 mutated and wild-type cancer cells would be very useful, because approximately 50% of cancer cells are p53 mutated and the other half are p53 wild type. Also, inactivation of p53 causes resistance to various cancer therapies including the use of an angiogenesis inhibitor (*13*) and 5-fluorouracil (*14*). The effect of mate saponin on p53 wild-type colonic cancer cells has not been reported. Thus, in the current study, we investigated the effects of mate saponins on cell growth and cell cycle arrest in p53 wild type colonic adenocarcinoma RKO cell lines which is known to be DNA mismatch repair defective, and p53 mutated colonic adenocarcinoma HT-29 cell lines.

We found that ursolic acid is the strongest anti-proliferative bioactive compound to both colon cancer cells. Ursolic and oleanolic acids have been shown to have a protective effect against colon carcinogenesis *in vivo* (*15*). Mate saponins are amphiphilic compounds and categorized as triterpenoic saponins as soy saponins which have been shown to be able to interact with the cancer cell membranes that are rich in phospholipids and cholesterol and with the hydroxyl groups on the aglycone moiety (*16*). Our results showed that chlorogenic acid has a weak inhibition to both HT-29 and RKO cells which is consistent with Park et al. (*17*) who indicated that chlorogenic acid did not protect against AOM-induced tumorigenesis. Moreover, we found that cisplatin inhibited HT-29 and RKO cell proliferation with IC_{50} values consistent with published data (*18–21*).

Yerba mate saponins reduced cell growth and caused cell cycle arrest in both cell lines. The influence of mate saponins on p21 was studied because of its suggested critical role in suppressing cell growth. Cell cycle is regulated by the activity of cyclin/cyclin-dependent kinase (CDK). This cyclin–CDK complex is regulated by CDK inhibitors such as p21 and p27. Mate saponins caused a dose-dependent increase in the expression of p21 and p27 in both HT-29 and RKO cells; p21 is well known as a p53 response gene capable of inhibiting multiple CDKs, resulting in the induction of G1 or G2 cell cycle arrest. Our results clearly demonstrated that G1 arrest via p21, by mate saponins, was through the upregulation of p53.

Mate saponins increased the amount of RKO and HT-29 cells undergoing apoptosis in a concentration-dependent manner. Members of the Bcl-2 family of proteins are critical regulators of the apoptotic pathway (*22*, *23*). These proteins consist of the major anti-apoptotic proteins, Bcl-x (L) and Bcl-2, and the major pro-apoptotic proteins Bax and Bak. Bax controls mitochondrial permeability and cytochrome c expression, and the release of cytochrome c from mitochondria to the cytoplasm is a key step in the initiation of apoptosis. As a downstream product of cytochrome c, caspases are critical mediators of the principal factors found in apoptotic cells (*24*). In the present study, mate saponins inhibited colon cancer cells proliferation by inducing apoptosis through increasing Bax/Bcl-2 ratio. These findings suggest that apoptosis induction in mate saponins-treated RKO cells involves the activation of the mitochondrial pathway. Consistent with our previous study, HT-29 cells treated with mate saponins resulted in a dose-dependent decrease in the anti-apoptotic Bcl-2 protein and increase in the expression of pro-apoptotic Bax protein (*10*). Our findings suggest the possible value of mate saponins against human colon cancer by promoting apoptosis of cancer cells.

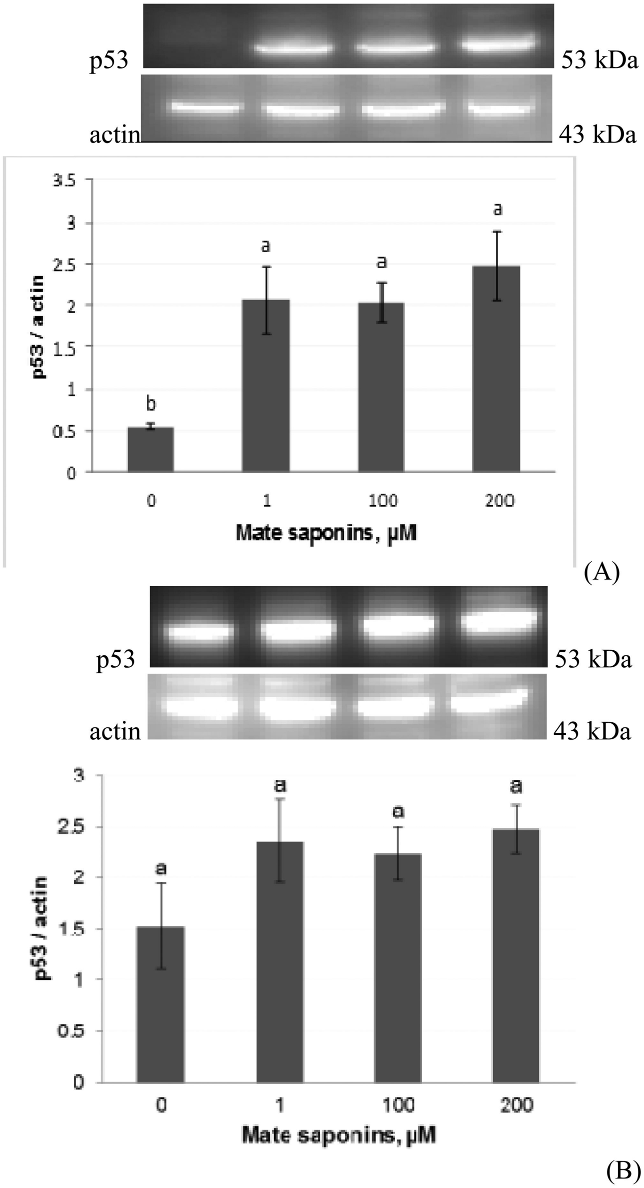

Figure 7. Effect of different concentrations of mate saponins on protein p53 expression in (A) HT-29 and (B) RKO assessed by Western blots. Actin was used as a protein loading control. The data represent the mean ± SD of a triplicate from three independent experiments. Different letters indicate significant differences, $p < 0.05$.

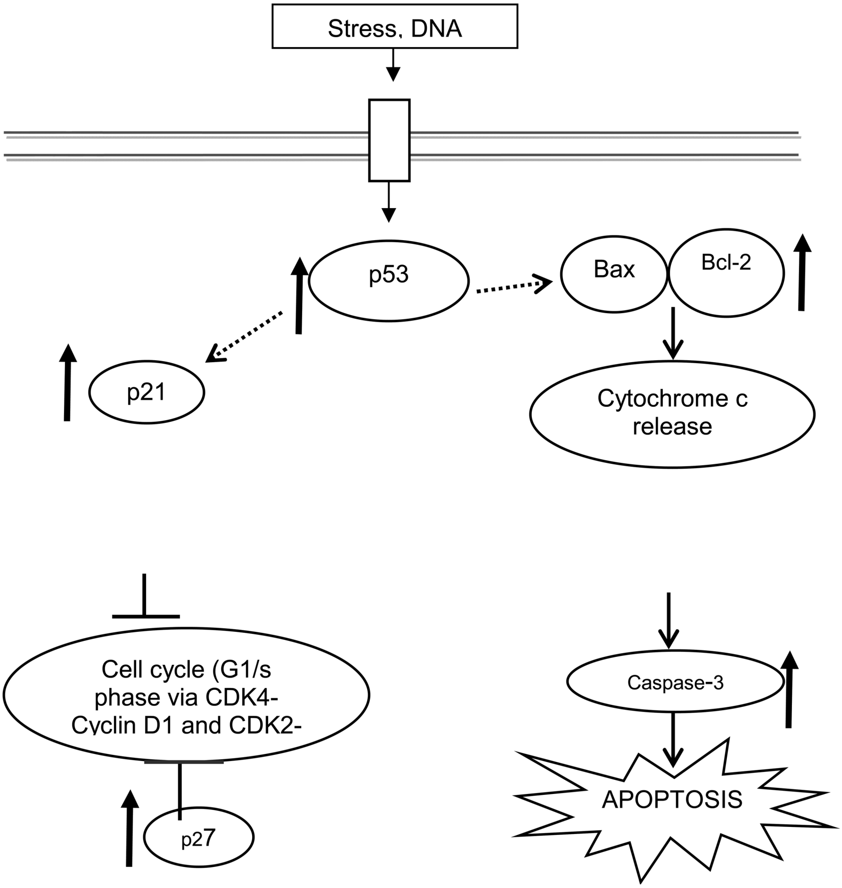

Figure 8. Proposed mechanism through which mate saponins induce apoptosis and cell cycle arrest via upregulating p53.

Cellular stress and DNA damage typically trigger the p53 tumor suppressor gene to mediate a series of antiproliferative strategies by inducing both cell cycle arrest and apoptosis. One important link between p53 and apoptosis is based on the transcriptional control of proapoptotic members of the Bcl-2 family, such as Bax. The relationship between p53 protein and the HT-29 cell death is still not clear (*25*). Shen et al. (*26*) have found that 2'-OH flavanone inhibits the growth of HT-29 cells via increasing the expression of p21, but it has no effect on p53 protein. Tsai et al. (*7*) did not find any inhibitory effect of soy saponin on the p53 protein of WiDr cells (wild type p53 human colon cancer cells), consistent with our results. The p53 protein expression was not affected by mate saponins treatment in RKO cells, which contain wild-type p53 protein but mate saponins induced p21 in this cell line. These results indicate that wild-type p53 is not involved in the mate saponins-induced apoptosis in colon cancer cells. This mechanism of mate saponins action is independent of the status of the p53 tumor suppressor gene. In addition, mate saponins still induced apoptosis in HT-29 cells, which contain mutant p53. We found that mate saponins could enhance the p53 cascade and prevent the expansion of mutated epithelial cells.

Understanding the mechanism by which mate saponin induces cell cycle arrest in colonic adenocarcinoma cells has the potential of providing information needed to prevent or reduce the growth of colonic tumors. This might provide an understanding of how mate saponins reduce colonic epithelial cell growth *in vitro*. Further study of yerba mate saponins on tumor growth *in vivo* is yet still needed. An explanation for this mechanism of mate saponins *in vitro* and *in vivo* may guide rational approaches for preventing colonic carcinogenesis in humans.

In summary, we proposed the mechanism by which yerba mate saponins inhibit HT-29 and RKO colon cancer cell proliferation by induction of cell cycle arrest and apoptosis via p53 cascade (Figure 8).

Yerba Mate saponins arrested G1 cell cycle by inducing p21 and p27 CDK inhibitors. Mate saponins induced mitochondrial apoptosis by increasing the expression of the pro-apoptotic protein Bax, and decreased the expression of anti-apoptotic protein Bcl-2, resulting in an increase in caspase-3 activity. In addition, the ability of mate saponins to suppress cell growth of colonic tumorigenic cells was independent of the p53 status of the cells. This eliminates the need to screen tumorigenic colonic tissue for p53 status before treatment with mate saponins. Our findings suggest the possible value of mate saponin against human colon cancer by inducing cell cycle arrest and promoting apoptosis; mate saponins might be an effective agent in the prevention of CRC.

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The authors declare no conflicts of interest.

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